INVESTIGATING THE ROBUSTNESS OF A STATISTICAL METHOD TO COMPARE MASS SPECTRA OF FENTANYL ISOMERS

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ABSTRACT

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The typical method for the identification of seized drugs is to analyze unknown samples using gas chromatography–mass spectrometry (GC-MS) and to perform a visual comparison of the resulting mass spectrum to a suitable reference spectrum. However, for spectra of structurally similar compounds, visual comparison of spectra for identification can be challenging. Previous work in our laboratory focused on the development of a statistical method to compare the mass spectrum of an unknown sample to a suitable reference spectrum using an unequal variance *t*-test.

In this work, GC-MS was used to analyze two sets of fentanyl isomers which included the *ortho*-, *meta*-, and *para*- forms of fluoroisobutyryl fentanyl (FIBF) and the *ortho*-, *meta*-, and *para*- forms of fluorobutyryl fentanyl (FBF). All compounds were analyzed over three months and the resulting spectra within each month were statistically compared. The ability to maintain correct association and discrimination across the three-month time study as well as the effects of refining the model on the overall results were observed. Proper association and discrimination of the FIBF and FBF spectra were achieved in most cases at the 99.9% confidence level and the ability to maintain similar overall results across the time study was demonstrated. Refining the model resulted in the reversal of an incorrect association (false positive) and a greater number of discriminating ions in many comparisons. Ultimately, this research provides insight into the robustness of the previously developed statistical comparison method to differentiate between positional isomers using instrumentation readily available in a forensic laboratory.

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I. Introduction

1.1 Fentanyl Epidemic

Fentanyl is a Schedule II synthetic opioid that has medical applications as a pain killer and as an anesthetic. This synthetic opioid is approximately 50 to 100 times more potent than morphine and is known to provide a euphoric high and to be very addictive.[1] Fentanyl was first synthesized in 1960 and approved for medical use by the Food and Drug Administration (FDA) in 1972. Very soon after its debut on the market, illicit fentanyl use began. In the late 1990s, the FDA issued warnings about the use of the drug and recommended that it only be prescribed to patients in a level of pain not managed by less potent opioids. The problem of illicit fentanyl use has only grown in the 2000s, with a dramatic increase in 2013.[2] According to the Drug Enforcement Administration's 2019 National Drug Threat Assessment, fentanyl is the main contributor to the ongoing opioid crisis and is expected to remain a serious threat to the United States in years to come.[3]

An additional problem to the growing fentanyl epidemic is that as soon as synthetic drugs become regulated under the Controlled Substances Act, new analogs of the regulated compound appear on the market. These analogs are synthesized to imitate the effects of the regulated compounds, but are sufficiently different structurally to evade legal ramifications. This has led to a fentanyl and fentanyl analog epidemic, with more than 77 fentanyl analogs classified as Schedule I substances. In 2016, fentanyl surpassed heroin as the drug most often involved in deadly overdoses. The number of deaths due to opioid overdoses involving fentanyl analogs almost doubled between 2016 and 2017, with around 14 analogs observed the most often. Among these main analogs are *para*-fluoroisobutyryl fentanyl (*p*-FIBF) and *para*-fluorobutyryl fentanyl (*p*-FBF). These two compounds are positional isomers of each other and distinction of isomers such as these can be challenging due to the high degree of structural similarity.[2] This research will focus on the two sets of positional isomers of FBF and FIBF.

Positional isomers are compounds that have the same core structure as well as the same chemical formula and molecular weight.[4] However, the difference is in the placement of the functional group(s) on the compound. As an example, the three positional isomers of FIBF (*ortho*-FIBF, *meta*-FIBF, and *para*-FIBF) have the same chemical formula of $C_{23}H_{29}FN_2O$ and the same molecular weight of 368 atomic mass units (amu). The only difference is the position of the fluorine substitution on the aniline ring – either in the *ortho* position, the *meta* position, or the *para* position.[5] Due to the high similarity in structure, it can be very difficult to distinguish positional isomers using the typical instrumentation used in forensic laboratories for seized drug identification.

1.2 Identification of Seized Drugs using Gas Chromatography-Mass Spectrometry

The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) has published recommendations for the identification of seized drugs.[6] As part of the recommendations, the analytical techniques typically used for identification are separated into three categories: A, B, and C. These categories are used to create an analytical scheme to be followed in order to ensure that the series of tests and techniques selected will offer enough selectivity and specificity for accurate identification. Category A techniques provide the highest level of selectivity through structural information. Such techniques include infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS). When a Category A technique is used as part of the analytical scheme, only one other technique from either Category A, B, or C is needed for identification. On the other hand, if a Category A technique is not used, three different techniques must be used, with at least two of those belonging to Category B which provides the second level of selectivity through chemical or physical characteristics. The typical method for the identification of controlled substances is to analyze samples with the use of a Category A technique (MS) coupled to a Category B technique, gas chromatography (GC).

For GC-MS analysis, a submitted sample is dissolved in a suitable solvent and injected into the GC. Following injection, the components of the sample are volatilized and separated via GC, providing chemical characteristics, then go on to the MS to be ionized, providing structural information. The results that are generated from this technique include a chromatogram with retention time information and a mass spectrum with nominal mass information.

To identify the seized drug present in the submitted sample, a visual comparison of the resulting mass spectrum to a suitable reference spectrum is conducted.[6] The reference spectrum may be a known standard analyzed on the same instrument under equivalent conditions or may be a result from a reputable mass spectral library such as the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health (NIST/EPA/NIH) Mass Spectral library. While the National Academy of Sciences (NAS) deems the identification of controlled substances to be a mature forensic discipline, there are some limitations to this method of analysis.[7] Identification is limited by the availability of pre-established mass spectral libraries, which is even more difficult when identifying synthetic analogs as well as structural and positional isomers. In addition, library search algorithms do not provide a measure of statistical confidence in the identification, which is desired by the NAS. Currently, only a visual assessment between the spectrum of the submitted sample and the reference spectrum is conducted. And finally, the acceptance criteria to determine how similar the spectra are for an identification may differ among laboratories and between cases.[8]

1.2.1 Gas Chromatography-Mass Spectrometry (GC-MS)

The most common technique for the identification of controlled substances is GC-MS. In order to use the method, the submitted sample must be dissolved in a suitable solvent prior to the injection into the GC. Following injection, the sample is vaporized into the gas phase and separated into its various components using a capillary column coated with a liquid stationary phase. An inert carrier gas propels the compounds through the column, and as they are separated based on volatility and affinity to the stationary phase, the components elute from the column at different times. Upon completion of separation via GC, the separated components move into the mass spectrometer through a transfer line that is heated to keep the sample in the gas phase.[9,10]

There are three main components to the mass spectrometer: the ionization source, the mass analyzer, and the detector. Once the separated components elute from the GC column, they are ionized in the ion source of the mass spectrometer. While there are many different types of ionization in MS, the most commonly used in seized drug analysis is electron ionization (EI), which is shown in Figure 1.1.[10] Ionization through EI involves the bombardment of the sample molecules with a high energy electron beam (70 eV). Produced by heating a wire filament with an electric current, the beam is attracted to a positive charge at the opposite end of the ionization chamber. The beam of electrons moves orthogonally to the transfer line and when the electrons and the gas-phase molecules from the transfer line come into proximity with one another, positive radical ions are formed.[10] This is possible because the energy of the electron beam (70 eV) is sufficiently high to break the bonds of most organic compounds (4-20 eV).[11] Following the formation of the positive radical ions, the positively charged repeller electrode repels the ions toward the negatively charged ion focusing plate, which acts to focus the ion beam before acceleration of ions into the mass analyzer.[10]

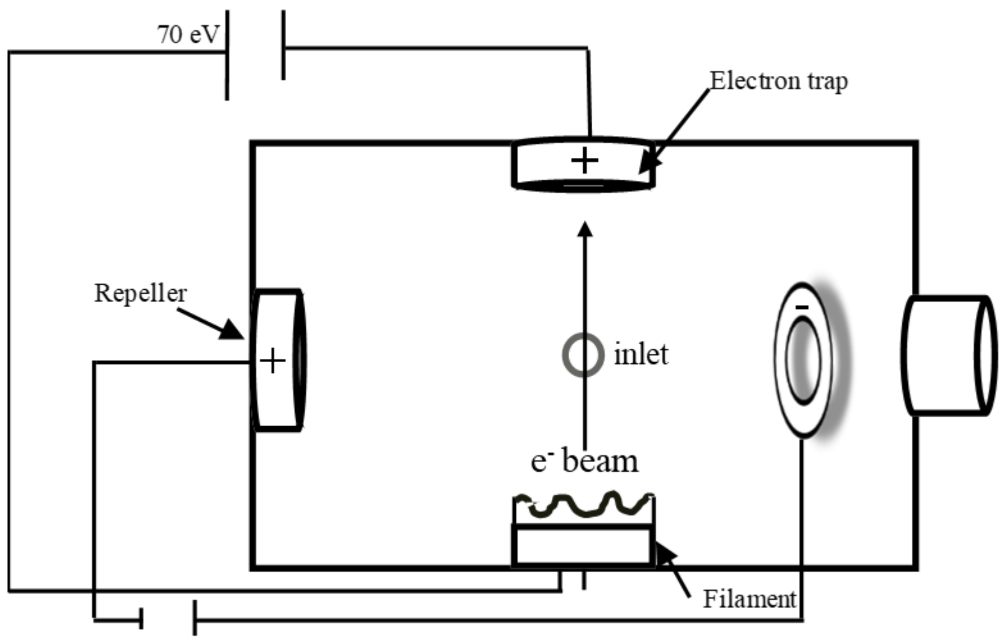

Figure 1.1 Diagram of an electron ionization source

There are also different types of mass analyzers; however, the most commonly used in benchtop GC-MS instruments is the single quadrupole mass analyzer. This type of mass analyzer consists of four cylindrical rods that are each set parallel to one another, as shown in Figure 1.2.[12] Each opposing rod pair is connected electrically, and a radio frequency (RF) voltage and direct current (DC) are applied between one pair of rods and the other. For a specific ratio of voltages, as the ions travel down the quadrupole, only those of a certain mass-to-charge (*m/z*) ratio will reach the detector, while all other ions will have unstable trajectories and collide with the rods. This allows for either the selection of a specific ion or the scanning of a range of *m/z* values by varying the applied voltage.[12]

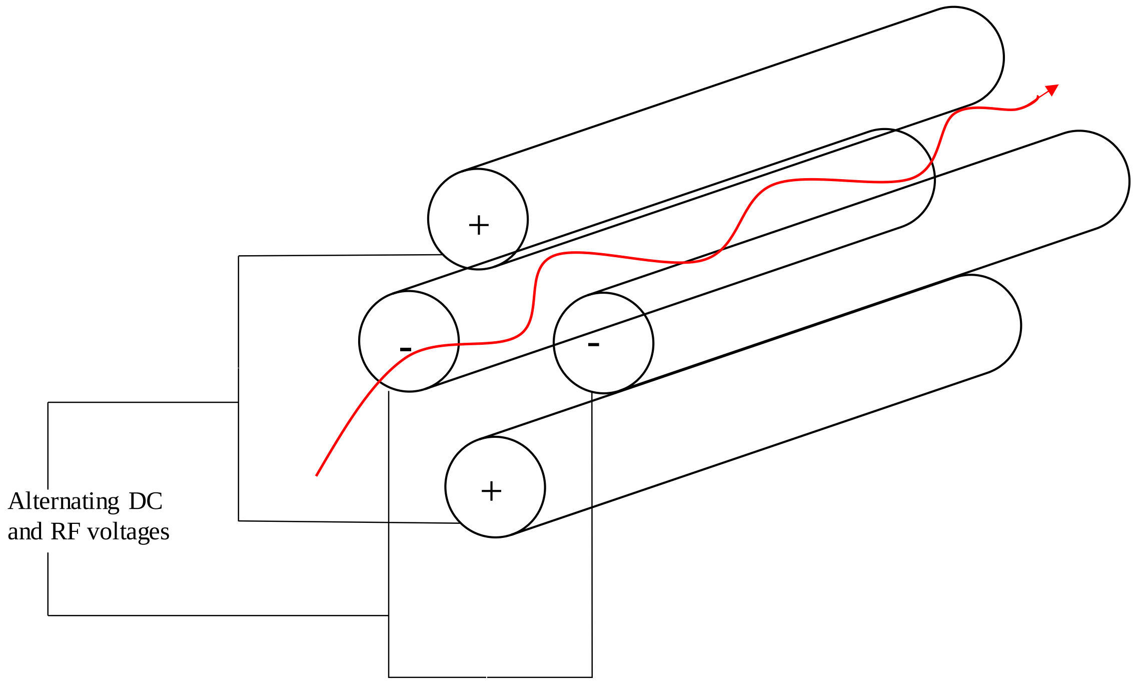

Figure 1.2 Diagram of a single quadrupole mass analyzer

The ions of a specific *m/z* value that successfully travel through the mass analyzer then reach the detector. In a bench-top GC-MS system, the most common type of detector is a continuous-dynode electron multiplier (EM), which is shown in Figure 1.3.[10] A continuous dynode system uses a horn-shaped funnel of glass coated with a thin film of semiconducting material. A negative high voltage is applied at the wider end and goes to a positive voltage at the narrow end. When the positively charged ions coming from the mass analyzer hit the EM, secondary electrons are emitted. Due to the electric potential being applied, the emitted electrons will accelerate to the next metal plate and induce emission of more secondary electrons. This process is repeated until a cascade of secondary electrons has been produced that results in amplification of the ion signal. The gain can range from 10^4-10^7. Once the signal has been amplified, the current is measured at that *m/z* value. This occurs at each *m/z* value within the scan range and the computer system attached to the detector converts the data into a mass spectrum.[13]

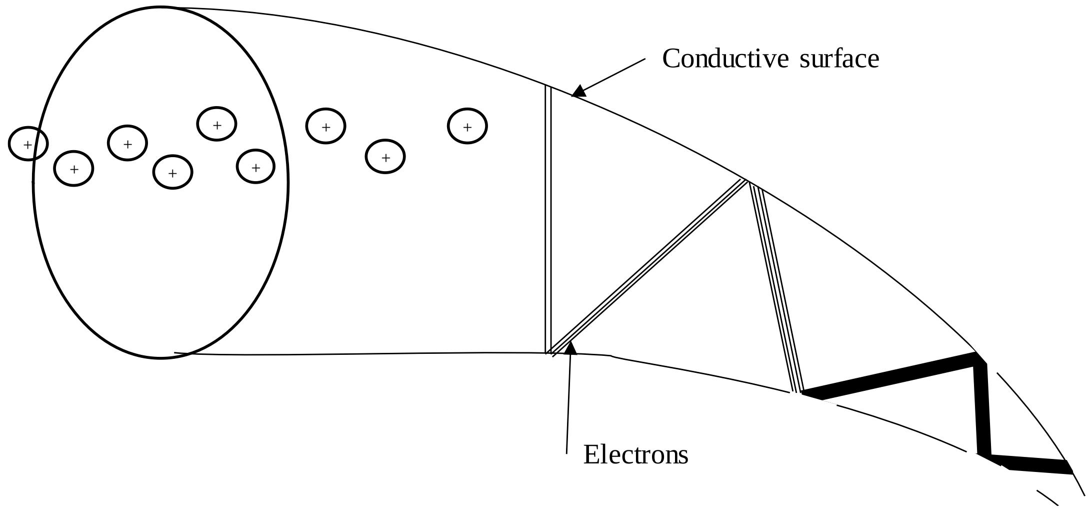

Figure 1.3 Diagram of continuous dynode electron multiplier

1.2.2 Gas Chromatography-Mass Spectrometry Limitations for NPS Analog Identification

In recent years, a challenge facing forensic drug analysts has been correctly identifying the increasing number of new analogs and isomeric forms of novel psychoactive substances (NPS), which includes fentanyl and related analogs. For some laboratories, the exact identity of a drug compound must be reported. This can be difficult as structural and positional isomers of drug compounds can co-elute during chromatography, have identical molecular weights, and often produce visually similar mass spectral fragmentation patterns – all parts of the analytical scheme that analysts use to identify controlled substances. To overcome these challenges, many research groups have developed other methods to distinguish isomers.

One avenue that has been investigated is the use of multivariate statistical methods to aid in the identification of positional isomers based on EI mass spectra. Bonetti used methods such as principal component analysis (PCA) and linear discriminant analysis (LDA) to differentiate isomers of fluoromethcathinone (FMC) and fluorofentanyl.[14] In this study, the mass spectra of three isomers of FMC and three isomers of fluorofentanyl were collected twice a day on six instruments over five days. An additional nineteen blind samples were also included. Visual inspection of the LDA plots was paired with objective classifications using posterior probabilities generated during LDA. Bonetti's conclusion was that the use of multivariate statistics is a feasible way to highlight small but reproducible differences in the mass spectra of positional isomers for identification purposes.[14]

In another study using multivariate statistics, Davidson and Jackson differentiated positional isomers of 1,5-dimethoxy-N-(N-methoxybenzyl)phenethylamines (NBOMes).[15] The isomers were differentiated based on retention indices and ion ratios of only the fifteen most abundant ions in the spectra using PCA and LDA. In conclusion, the LDA classification was 99.5% accurate across different instruments and was 99.9% accurate when using the same instrument.[14] While both of the studies from Bonetti and Davidson and Jackson provide very useful methods to identify and differentiate positional isomers using GC-MS, the methods required the use of several instruments and different compounds to develop the robust training sets necessary to perform multivariate statistical analysis. This can be very time-consuming and difficult in a forensic laboratory setting.[14,15]

Other methods to distinguish positional isomers include the use of different GC detectors rather than, or in addition to, MS. Kranenburg *et al.* reported the use of vacuum-ultraviolet spectroscopy (VUV) as a detector for GC to differentiate isomers of phenethylamines and cathinones.[16] The GC-VUV system provided spectra with distinct differences for positional isomers of substituents on aromatic rings. Although the VUV spectra of some classes of drug compounds appeared visually similar, small differences were enough to differentiate isomers because of the robustness and reproducibility of the spectral data.[16]

Other methods for positional isomer differentiation include modifications to the ionization method, which is generally EI. One such modification, reported by Kranenburg *et al.* is low-energy EI, which can lead to changes in intensity ratio patterns which affect each positional isomer differently.[17] Using an ionization energy of 15 eV (rather than the more conventional 70 eV), mass spectra of cathinone isomers were distinguished with the aid of PCA and LDA. The accuracy of this method was demonstrated with 100% correct isomer identification of six forensic case samples.[17]

Another modification to the ionization method which was reported by Buchalter *et al*. was the use of GC with tandem cold EI-MS and VUV detection.[18] Cold EI-MS is based on cooling the molecules as they are transported from the GC into the mass spectrometer. Reducing the temperature of the molecules enhanced the survival of the ions during ionization. The study investigated the efficacy of the tandem detection system for the analysis of twenty-four fentanyl analogs, including seven sets of positional isomers. In conclusion, the combination of GC in tandem with cold EI-MS and VUV was determined to result in higher confidence in sample identification using retention time and mass spectra that included larger relative intensities of the molecular ion. While the positional isomers were found to produce very similar mass spectra even with cold EI-MS, the VUV spectra were unique enough for distinguishability in this case. While the methods presented by Kranenburg *et al.* and Buchalter *et al.* did allow for the distinction of isomers, the instrumentation is not widely available in forensic laboratories and would be expensive to institute.[16,17,18]

1.3 Statistical Comparison Method

To address limitations in positional isomer differentiation, Willard *et al*. developed a statistical method to compare the mass spectrum of an unknown sample to that of a reference material using an unequal variance *t*-test.[8,19] In this approach, *t*-tests are used to statistically compare the mean abundances at every corresponding *m/z* value in the two spectra. The null (H_0) and alternative (H_a) hypotheses are shown below in Equations 1.1 and 1.2, respectively

$$H_0 : \left|\bar{x}_{1j} - \bar{x}_{2j}\right| = 0 \tag{1.1}$$

$$H_a : \left|\bar{x}_{1j} - \bar{x}_{2j}\right| \neq 0 \tag{1.2}$$

where $\bar{x}_{1j}$ and $\bar{x}_{2j}$ are the mean abundances of ion *j* in spectra 1 and 2. The hypotheses are tested using the Welch's *t*-test calculation (t_{calc}) as shown in Equation 1.3

$$t_{\text{calc}} = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{s_1^2}{n_1} - \frac{s_2^2}{n_2}}} \tag{1.3}$$

where $\bar{x}_1$and $\bar{x}_2$ are the mean abundances at a common *m/z* ratio for the two spectra and n_1 and n_2 are the number of spectra used to calculate the standard deviations (s_1 and s_2) of the mean abundances. The degrees of freedom calculation for the *t*-test is shown in Equation 1.4

$$v = \frac{\left(\frac{s_1^2}{n_1} - \frac{s_2^2}{n_2}\right)}{\frac{1}{n_1-1}\left(\frac{s_1^2}{n_1}\right)^2 + \frac{1}{n_2-1}\left(\frac{s_2^2}{n_2}\right)^2} \tag{1.4}$$

In order to perform the *t*-test, a critical *t*-value is determined using the appropriate statistical table according to the degrees of freedom which were calculated and the user-specified confidence level. The calculated *t*-value is then compared to the corresponding critical *t*-value. If H_0 is accepted at every *m/z* value, the two spectra are determined to be statistically indistinguishable, at the confidence level specified by the user when performing the *t*-test. However, if H_a is accepted at any *m/z* value, then the two spectra are determined to be statistically distinguishable.

Through hypothesis testing, the association (or lack thereof) between the two spectra in question can be determined.

The unequal variance *t*-test calculations are performed in an Excel spreadsheet that is automated to perform the calculations and report whether the spectra are statistically indistinguishable or statistically distinguishable. In cases where statistical discrimination is observed, the number and identity of the discriminating ions are recorded. In cases where statistical similarity is observed, a maximum and a minimum random-match probability (P_{max} and P_{min}, respectively) are automatically calculated to estimate the probability that the fragmentation pattern observed in the spectra under comparison occurred by random chance alone (Eq. 1.5)

$$P = \prod_{j=(m/z)_i}^{(m/z)_f} P_j = P_{(m/z)_i} \times P_{(m/z)_{i+1}} \times \cdots \times P_{(m/z)_f} \qquad (1.5)$$

where $(m/\mathrm{z})_{\mathrm{i}}$ is the initial mass-to-charge ratio and $(m/z)_{\mathrm{f}}$ is the final mass-to-charge ratio in the mass scan range.[7,11] The frequency of ion occurrence was determined from the NIST Mass Spectral Search Program.[19,20] The P_{min} is calculated assuming that the occurrence of each ion is a random and independent event; whereas, the P_{max} is calculated assuming that the occurrence of every ion is a dependent event. The P_{max} and P_{min} are calculated using the multiplicative rule and ions that are known to be common contaminants from column and septum degradation and ions from the mass calibrant are excluded from the calculations if they fall below 5% relative abundance of the base peak. These ions include *m/z* 69, 73, 147, 207, 219, 221, 281, 295, and 355.[8,19]

Also part of the automated method is the calculation of Pearson product-moment correlation (PPMC) coefficients. This allows for another measure of spectral similarity between the two spectra being compared. The calculation is shown in Equation 1.6

$$r_{1,2} = \frac{\sum_{j=(m/z)_i}^{(m/z)_f} (x_{1j} - \bar{x}_1)(x_{2j} - \bar{x}_2)}{\sqrt{\sum_{j=(m/z)_i}^{(m/z)_f} (x_{1j} - \bar{x}_1)^2 (x_{2j} - \bar{x}_2)^2}} \tag{1.6}$$

where $r_{1,2}$ is the PPMC coefficient between spectrum 1 and spectrum 2, x_{1j} and x_{2j} are the abundances of ion *j* in each spectrum, $\bar{x}_1$ and $\bar{x}_2$ are the mean abundances of all ions in spectrum 1 and 2, respectively, between the initial $(m/z)_i$ and final $(m/z)_f$ mass-to-charge values in the scan range. With a range between +1 to -1, PPMC coefficients demonstrate either a positive or negative correlation between the two spectra under comparison. There are four ranges of correlation: strong correlation ($r > \pm0.80$), moderate correlation ($\pm0.50 < r < \pm0.79$), weak correlation ($r < \pm0.50$), and no correlation (r close to zero).

In order to consistently and uniformly represent instrumental variation while performing this statistical comparison method, a mathematical model was developed to predict standard deviations. The response of the electron multiplier detector in the mass spectrometer is based on counting statistics, which can be used to predict the standard deviation of an ion with known abundance. To model the electron multiplier response, a set of samples is analyzed in replicate at different concentrations, and representative mass spectra are generated on the instrument. The mean abundance of each *m/z* value is determined with the associated standard deviation and these values are plotted on a logarithmic scale. Linear regression is then performed and the resulting regression coefficients are used to predict the standard deviation of ions analyzed on that specific instrument. The predicted standard deviations are independent of the identity of the compound, concentration, injection volume, and split ratio. The plot of standard deviation versus mean abundance and following linear regression analysis needs to be re-evaluated regularly and re-defined following major maintenance that requires venting the system.[8,20]

1.3.1 Previous Applications of the Statistical Comparison Method

The statistical comparison method was developed and validated for a set of normal alkanes and has been applied for the differentiation of amphetamine-type stimulants and salvinorins extracted from the plant material, *Salvia divinorum*.[8,19,20,21] More recently, application of the method to successfully discriminate positional isomers of fluoromethamphetamine and ethylmethcathinone has been demonstrated,[22] along with an initial investigation into the effects of instrument parameters (tune and split ratio) on the statistical association and discrimination of isomers.[23] In this study, spectra were collected on consecutive days and then about a month apart to be compared and successful association and discrimination of the positional isomers was generally observed. In addition to the research involving statistical comparisons of various samples, the method used to predict standard deviation based on electron multiplier counting statistics was further investigated. Typically, a linear regression plot of standard deviation and mean abundance is used; however, during the investigation, it appeared that there were two separate linear regions that could be used.

1.4 Research Objectives

The main objective in this research was to investigate the robustness of the previously developed statistical comparison method for differentiation of positional isomers. To achieve this objective, two sets of fentanyl isomers (FIBF and FBF) were analyzed on the same instrument under equivalent conditions. With the chosen sets of fentanyl isomers being not only positional isomers, but also structural isomers of one another, the ability of the method to differentiate was tested in ways not previously investigated. As mass spectral data were collected for each isomer, the robustness of the method was further assessed by comparing spectra collected across a three-month time period. During this relatively short time study, the effects of major instrument maintenance (involving venting of the system) as well as high instrument usage (involving other research groups using the same instrument for other purposes) on the ability to maintain proper association and discrimination of the fentanyl isomers were investigated.

In addition, the method to predict standard deviation based on the electron multiplier response was further refined. Previous research investigated the possibility of two linear regions within the regression instead of just one region. In this work, following comparisons that resulted in inaccurate association and discrimination of isomers, the method to predict standard deviation utilizing two linear regions of the regression was tested. The effect of the refined method on the ability to successfully associate and discriminate the isomers was then investigated.

By applying the statistical comparison method to a new set of both structural and positional isomers, the robustness of the method for the use of isomer and analog differentiation will be further investigated and the accuracy to which isomers are successfully associated and discriminated will be determined. Following the refinement of the method to more accurately predict the standard deviations, this method of positional isomer differentiation can be compared to additional methods for use in forensic science laboratories.

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II. Materials and Methods

2.1 Preparation of Fentanyl Analog and Isomer Solutions

The *ortho*-, *meta*-, and *para*- isomers of fluoroisobutyryl fentanyl (FIBF) and *ortho*-, *meta*-, and *para*- isomers of fluorobutyryl fentanyl (FBF) were purchased from Cayman Chemical (Ann Arbor, MI). Each compound was prepared at 1 mg/mL in methanol (ACS Grade, Sigma Aldrich, St. Louis, MO) prior to analysis.

2.2 Gas Chromatography-Electron Ionization-Mass Spectrometry Analysis

Each isomer was analyzed using an Agilent 7890A gas chromatograph coupled to an Agilent 5975c mass spectrometer with triple axis detector and a CTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). The carrier gas was ultra-high purity helium (Airgas, Independence, OH) at a nominal flow rate of 1 mL/min. An inert GC capillary column was used with a 5% diphenyl-95% dimethylpolysiloxane stationary phase (VF-5ms, 30 m x 0.25 mm x 0.25 µm, Agilent Technologies).

Each isomer was analyzed over a three-month time period under two scenarios – to be used as the reference spectrum, which would typically be considered the reference standard in a forensic laboratory, and to be used as the comparison spectrum, or case sample if the method is applied to real casework. In the case of the reference spectrum analysis, each isomer was analyzed in replicate (n = 5) and in the case of the comparison spectrum analysis, each of the six isomers was analyzed once. One exception was during the first month collection, where only the three FIBF isomers were analyzed for comparison spectra and analysis was performed in replicate (n=3).

Each isomer was injected onto the instrument (1 µL) at a split ratio of 100:1 for each day of reference and comparison spectra data collection. Samples were all analyzed under equivalent conditions based on the following parameters. The injector port temperature was 220 °C and the oven temperature program was as follows: 200 °C for 1 min, 30 °C/min to 300 °C, with a final hold of 8 min. The transfer line was maintained at 300 °C and the mass spectrometer was operated in electron ionization mode (70 eV), with a scan range of *m/z* 40-450 and a scan rate of 4.51 scans/s.

2.3 Predicted Standard Deviation

In order to perform unequal variance *t*-tests at each *m/z* value between two mass spectra, the mean abundance and standard deviation of each abundance at every *m/z* value must be calculated (Equations 1.3 and 1.4, Section 1.3). Instead of calculating the mean abundance and standard deviation using replicates, the standard deviation can be predicted based on the counting statistics of the electron multiplier detector in the GC-MS. This method also allows for a consistent and uniform representation of instrumental variation while performing the statistical comparison method.

2.3.1 Modeling the Electron Multiplier Response

There are three different sources of noise in electron multipliers: background noise, shot noise, and proportional noise.[1] Representations of a signal dominated by each source of noise is shown in Figure 2.1. Background noise is constant and can be caused by a multitude of sources including the carrier gas and column from the gas chromatograph or even a vacuum leak in the mass spectrometer. Shot noise is caused by the randomness in the number of electrons that are multiplied throughout the continuous dynode. Each electron that strikes the dynodes results in a random three to 6 electrons multiplied. Shot noise is proportional to the square root of the signal. Proportional noise scales directly with the signal. The total noise which is observed for any given signal is from a combination of all three sources. The variances of each source of noise depend on the magnitude of the signal and the variance from all independent sources of noise are additive.[2]

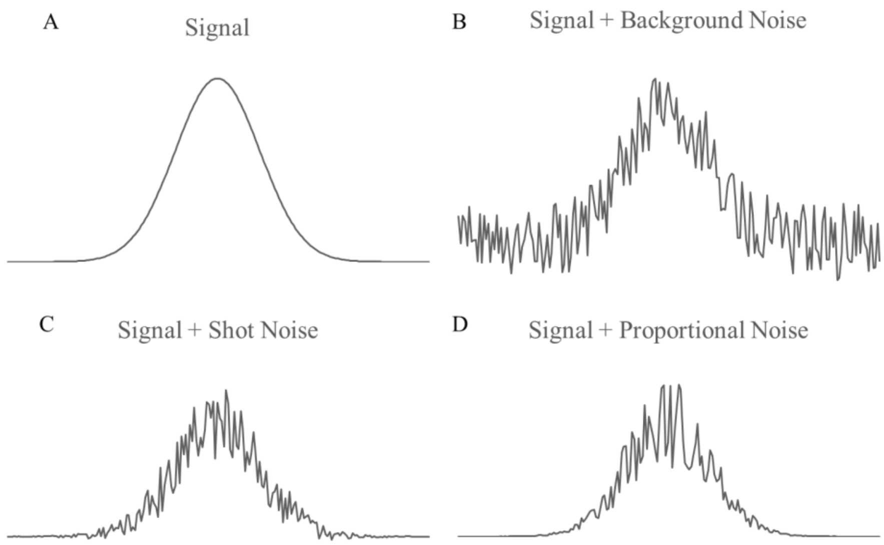


Figure 2.1 Representations of (A) a signal and one that is dominated by (B) background noise, (C) shot noise, and (D) proportional noise.

2.3.2 Preparation of Alkane Mixtures

In order to collect the data necessary to predict standard deviations on the instrument that was being used, a stock solution that included a mixture of four alkanes – *n*-heptane (C_7), *n*-decane (C_{10}), *n*-tridecane (C_{13}), and *n*-heptadecane (C_{17}) – was prepared. The alkanes were purchased from Sigma Aldrich, St. Louis, MO, USA. The stock solution was prepared by adding 0.5 mL of each alkane to a volumetric flask and diluting the solution up to 25 mL using dichloromethane (ACS grade, Macron Fine Chemicals, Darmstadt, Germany). This resulted in different concentrations of each alkane in the stock solution: 0.14 M C_7, 0.10 M C_{10}, 0.082 M C_{13}, and 0.065 M C_{17}. The stock solution was then diluted to four different concentrations: 75%, 50%, 25%, and 10%, which resulted in four samples (alkane mix 1-4) containing four alkanes each. This preparation process was repeated as necessary during the three months of data collection.

All four samples of the alkane mix were analyzed on the same GC-MS instrument in triplicate using parameters described by the National Center for Forensic Science. Each sample was injected with a volume of 1 μL at a 50:1 split ratio. The injector port temperature was maintained at 250 °C and the oven temperature program was as follows: initial temperature 50 °C held for 3 min, 10 °C/min to 280 °C, with a final hold of 4 min. The transfer line was maintained at 280 °C and the mass spectrometer was operated in electron ionization mode (70 eV), with a scan range of *m/z* 40 – 450, and a scan rate of 4.59 scans/s. Spectra were collected for each alkane in each concentration mixture for every replicate yielding a total of 48 spectra. This procedure was repeated each time major maintenance requiring venting of the instrument (*e.g.*, changing the column or cleaning the ion source) was performed.

2.3.3 Generation of Standard Deviation Plot

After data collection (48 total spectra), the mean abundances of the spectra collected at the apex of the chromatographic peak and the associated standard deviations for the replicates of each alkane at each concentration were calculated. A logarithmic plot of the standard deviation versus mean abundance was generated and linear regression analysis was performed in Microsoft Excel (version 12.0, Microsoft Corporation, Redmond, WA). The resulting slope and y-intercept from the regression equation were used to determine the predicted standard deviation of compounds analyzed on that specific instrument under equivalent conditions as long as the abundance of all ions are known. The predicted standard deviations are independent of the identity of the compound, concentration, injection volume, and split ratio, but are not independent of instrument. Therefore, separate plots must be produced if analyzing samples on different instruments and after venting of the system.

Due to the rigorous use of the Agilent instrument used for this research and the number of column changes during the duration of this research, a new regression plot was required for each month's analysis. Using a procedure to statistically compare the slopes of two regression lines detailed by Andrade and Estévez-Pérez, the slopes of each regression line were statistically compared on a month-to-month basis.[3]

2.4 Data Analysis

Representative mass spectra for the FIBF and FBF isomers were collected at the apex of the corresponding chromatographic peak (100% relative abundance). The data were exported into a CSV file from ChemStation (version #E.02.01.1177, Agilent Technologies) and transferred to a Microsoft Excel worksheet that is used to automate the previously developed statistical comparison method (Appendix Tables A2.1 – 2.3).[4,5]

The arrangement of the statistical comparison worksheet allows for the comparison of a single mass spectrum, the comparison spectrum in this case, to three replicate spectra, the reference spectra in this case. Mass spectral data for each isomer were collected across three consecutive months (September – November), which will be referred to as Month 1, Month 2, and Month 3. The specific days in which the samples were analyzed are shown in Figure 2.1. During Month 1, comparison spectra were collected twice (A and B) to observe the differences and similarities in the comparison results within the same month of data collection. Month 1A comparison spectra only included the three FIBF samples, whereas the Month 1B collection included all six isomers. In addition, reference spectra for the FBF compounds were collected on a separate day than the reference spectra for the FIBF compounds. This was the case for each month due to time constraints. During Month 2 and Month 3, comparison spectra were collected once for all six isomers and the reference spectra were collected in triplicate on a separate day. Collecting data in this manner ensured that comparisons were between spectra collected on different days, rather than comparisons of instrument replicates.

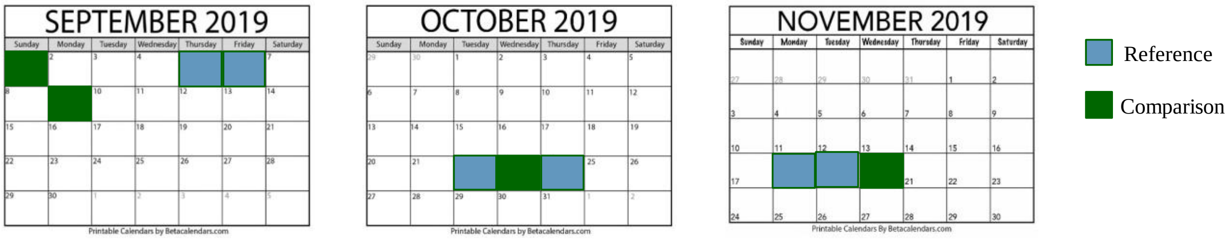


Figure 2.2 Representation of the days in which Spectrum 1 (blue) and Spectrum 2 (green) data were collected across September, October, and November.

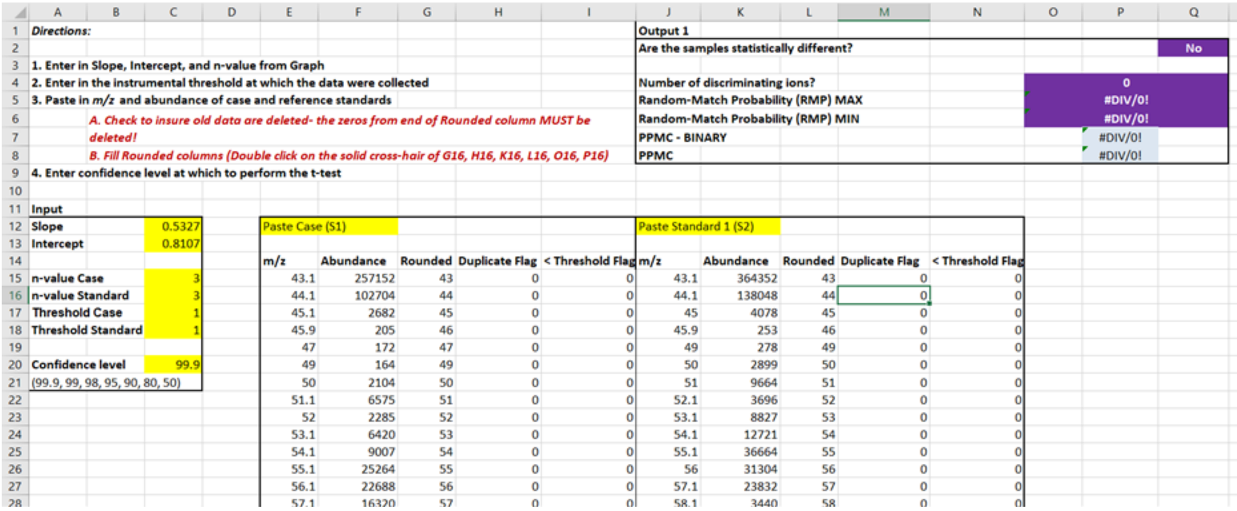
Once the raw mass spectral data were transferred into the Excel worksheet, the template automatically zero-filled and normalized the spectral data to ensure an abundance was given at each *m/z* value in the defined scan range of *m/z* 40-400 and that relative abundances could be statistically compared. The comparison worksheet was also automated to round each *m/z* value to the nearest whole number and to flag any duplicates. If two *m/z* values round to the same whole number, the second abundance is always used unless action is taken by the analyst. Within the worksheet, statistical comparisons were made by performing an unequal variance *t*-test at each *m/z* value in the scan range. These calculations were also automated by the worksheet and use the predicted standard deviation procedure discussed in Section 2.3. Taking the results of the *t*-test, the calculated *t*-values (t_{calc}) were compared to the critical *t*-values (t_{crit}) at each *m/z* value to determine the statistical similarity or dissimilarity of the two spectra under comparison. If t_{calc} is determined to be less than or equal to t_{crit} (and the null hypothesis is accepted) at every single ion, the two spectra being compared are considered statistically similar to one another. However, if t_{calc} is greater than t_{crit} (and the null hypothesis is rejected) at any single *m/z* value, the two spectra are considered statistically distinguishable.

In cases where statistical discrimination is observed, the number and identity of the discriminating ions are recorded. In cases where statistical similarity is observed, a random-match probability (*P*) is automatically generated to estimate the probability that the fragmentation pattern observed in the spectra under comparison occurred by random chance alone. Additionally, ions that are known contaminants from column and septum degradation as well as from mass tuning are automatically excluded from the statistical comparison worksheet if they are under the specified threshold.

Within the comparison method, a Pearson product-moment correlation (PPMC) coefficient is calculated to provide an additional numerical representation of spectral similarity between the comparison spectrum and reference replicate spectra. PPMC coefficients between replicates of each isomer as well as between each isomer were also calculated using the correlation function within the Analysis ToolPak Microsoft Excel add-in (Equation 1.6, Section 1.3).

Through this procedure of sample preparation, spectra collection, and data analysis, the sets of fentanyl isomers were compared using the statistical comparison method and the association and discrimination results were observed.

APPENDIX



Directions:

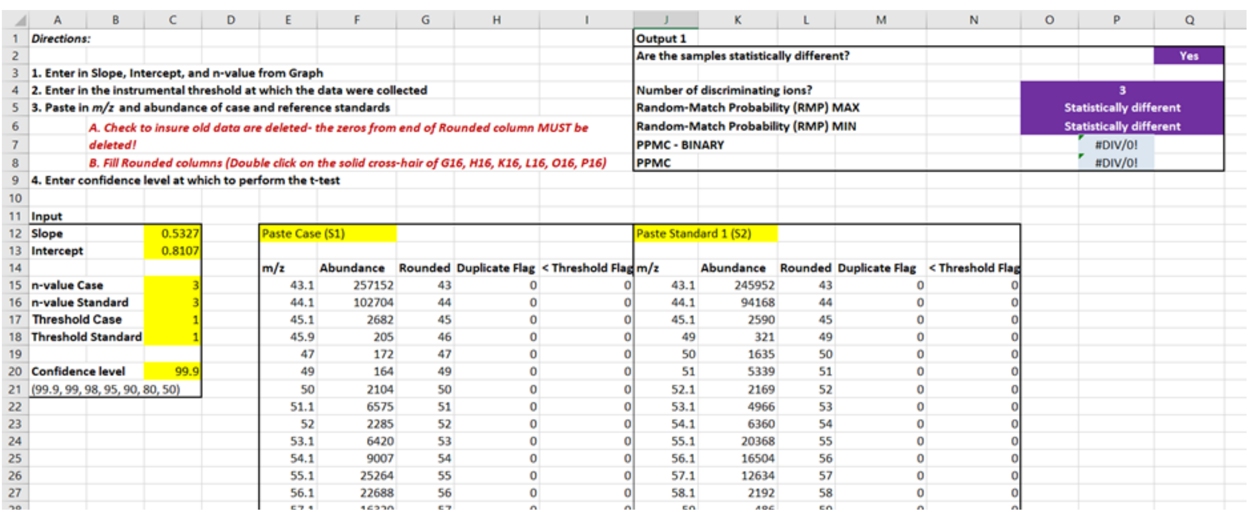
1. Enter in Slope, Intercept, and n-value from Graph
2. Enter in the instrumental threshold at which the data were collected
3. Paste in *m/z* and abundance of case and reference standards
A. Check to insure old data are deleted- the zeros from end of Rounded column MUST be deleted!
B. Fill Rounded columns (Double click on the solid cross-hair of G16, H16, K16, L16, O16, P16)
4. Enter confidence level at which to perform the t-test

Output 1	
Are the samples statistically different?	No
Number of discriminating ions?	0
Random-Match Probability (RMP) MAX	#DIV/0!
Random-Match Probability (RMP) MIN	#DIV/0!
PPMC - BINARY	#DIV/0!
PPMC	#DIV/0!

Input	
Slope	0.5327
Intercept	0.8107
n-value Case	3
n-value Standard	3
Threshold Case	1
Threshold Standard	1
Confidence level	99.9
(99.9, 99, 98, 95, 90, 80, 50)	

Paste Case (S1)					Paste Standard 1 (S2)				
m/z	Abundance	Rounded	Duplicate Flag	< Threshold Flag	m/z	Abundance	Rounded	Duplicate Flag	< Threshold Flag
43.1	257152	43	0	0	43.1	364352	43	0	0
44.1	102704	44	0	0	44.1	138048	44	0	0
45.1	2682	45	0	0	45	4078	45	0	0
45.9	205	46	0	0	45.9	253	46	0	0
47	172	47	0	0	49	278	49	0	0
49	164	49	0	0	50	2899	50	0	0
50	2104	50	0	0	51	9664	51	0	0
51.1	6575	51	0	0	52.1	3696	52	0	0
52	2285	52	0	0	53.1	8827	53	0	0
53.1	6420	53	0	0	54.1	12721	54	0	0
54.1	9007	54	0	0	55.1	36664	55	0	0
55.1	25264	55	0	0	56	31304	56	0	0
56.1	22688	56	0	0	57.1	23832	57	0	0
57.1	16320	57	0	0	58.1	3440	58	0	0

Figure A2.1 Statistical comparison Microsoft Excel template example for association



Directions:

1. Enter in Slope, Intercept, and n-value from Graph
2. Enter in the instrumental threshold at which the data were collected
3. Paste in *m/z* and abundance of case and reference standards
A. Check to insure old data are deleted- the zeros from end of Rounded column MUST be deleted!
B. Fill Rounded columns (Double click on the solid cross-hair of G16, H16, K16, L16, O16, P16)
4. Enter confidence level at which to perform the t-test

Output 1	
Are the samples statistically different?	Yes
Number of discriminating ions?	3
Random-Match Probability (RMP) MAX	Statistically different
Random-Match Probability (RMP) MIN	Statistically different
PPMC - BINARY	#DIV/0!
PPMC	#DIV/0!

Input	
Slope	0.5327
Intercept	0.8107
n-value Case	3
n-value Standard	3
Threshold Case	1
Threshold Standard	1
Confidence level	99.9
(99.9, 99, 98, 95, 90, 80, 50)	

Paste Case (S1)					Paste Standard 1 (S2)				
m/z	Abundance	Rounded	Duplicate Flag	< Threshold Flag	m/z	Abundance	Rounded	Duplicate Flag	< Threshold Flag
43.1	257152	43	0	0	43.1	245952	43	0	0
44.1	102704	44	0	0	44.1	94168	44	0	0
45.1	2682	45	0	0	45.1	2590	45	0	0
45.9	205	46	0	0	49	321	49	0	0
47	172	47	0	0	50	1635	50	0	0
49	164	49	0	0	51	5339	51	0	0
50	2104	50	0	0	52.1	2169	52	0	0
51.1	6575	51	0	0	53.1	4966	53	0	0
52	2285	52	0	0	54.1	6360	54	0	0
53.1	6420	53	0	0	55.1	20368	55	0	0
54.1	9007	54	0	0	56.1	16504	56	0	0
55.1	25264	55	0	0	57.1	12634	57	0	0
56.1	22688	56	0	0	58.1	2192	58	0	0

Figure A2.2 Statistical comparison Microsoft Excel template example for discrimination

Output 2

Discriminating ions

N Sample 1 (Case): 3

N Sample 2 (Standard): 3

Product: **0**

BZ	CA	CB	CC	CD	CE	CF	CG	CH	CI	CJ	CK	CL	CM	CN	CO
	Case		**Standard**												
m/z	**SD**	**Mean**	**SD**	**Mean**	**Tcalc**	**Tcalc2**	**w1**	**w2**			**DOF1**	**DOF2**		**Tcrit**	**Stat diff**
40	1.871E-05	0.00014	0.000188	0.00441	39.07518	39.07518	1.16656E-10	1.18135E-08	6.80428E-21	6.97799E-17	2	2	2	31.5991	0
41	0.0021326	0.10038	0.001648	0.09007	6.627942	6.627942	1.51594E-06	9.05469E-07	1.14903E-12	4.09937E-13	3	3	3	12.924	1
42	0.0035711	0.20562	0.002858	0.19366	4.528824	4.528824	4.25091E-06	2.72244E-06	9.03513E-12	3.70584E-12	3	3	3	12.924	1
43	0.0046449	0.29639	0.003227	0.2293	20.54638	20.54638	7.19175E-06	3.47095E-06	2.58606E-11	6.02376E-12	3	3	3	12.924	0
44	0.003939	0.23566	0.002594	0.16927	24.37942	24.37942	5.1718E-06	2.24337E-06	1.33738E-11	2.51636E-12	3	3	3	12.924	0
45	0.0002878	0.00619	0.000191	0.0045	8.506844	8.506844	2.76104E-08	1.21633E-08	3.81166E-16	7.39731E-17	3	3	3	12.924	1
46	2.415E-05	0.0002	1.92E-05	0.00018	0.718782	0.718782	1.94456E-10	1.23254E-10	1.89066E-20	7.59574E-21	3	3	3	12.924	1
47	2.234E-05	0.00018	1.44E-05	0.00012	3.531245	3.531245	1.6637E-10	6.87119E-11	1.38394E-20	2.36066E-21	3	3	3	12.924	1
48	1.898E-05	0.00014	1.56E-05	0.00014	0.241457	0.241457	1.20025E-10	8.08792E-11	7.20305E-21	3.27072E-21	3	3	3	12.924	1
49	4.226E-05	0.00043	2.4E-05	0.00025	6.349821	6.349821	5.95423E-10	1.92383E-10	1.77264E-19	1.85056E-20	3	3	3	12.924	1
50	0.0001744	0.00309	0.000138	0.00285	1.798915	1.798915	1.01381E-08	6.32837E-09	5.13902E-17	2.00241E-17	3	3	3	12.924	1
51	0.000372	0.00885	0.000298	0.00836	1.773744	1.773744	4.61315E-08	2.96788E-08	1.06406E-15	4.40415E-16	3	3	3	12.924	1
52	0.0002091	0.00397	0.00015	0.00323	5.022884	5.022884	1.45806E-08	7.5434E-09	1.06297E-16	2.84514E-17	3	3	3	12.924	1
53	0.0003836	0.00923	0.000312	0.00891	1.129438	1.129438	4.90411E-08	3.25278E-08	1.20252E-15	5.29029E-16	3	3	3	12.924	1
54	0.0005232	0.01422	0.000404	0.01274	3.890397	3.890397	9.12416E-08	5.4355E-08	4.16251E-15	1.47724E-15	3	3	3	12.924	1
55	0.0010784	0.03889	0.000862	0.03654	2.938145	2.938145	3.87626E-07	2.47472E-07	7.51268E-14	3.06212E-14	3	3	3	12.924	1
56	0.0009543	0.03281	0.000777	0.03165	1.621129	1.621129	3.03564E-07	2.01293E-07	4.60757E-14	2.02594E-14	3	3	3	12.924	1
57	0.0008137	0.02629	0.000624	0.02331	5.031327	5.031327	2.2072E-07	1.29612E-07	2.43586E-14	8.39969E-15	3	3	3	12.924	1
58	0.0002499	0.00509	0.00016	0.00352	9.177232	9.177232	2.08146E-08	8.53957E-09	2.16625E-16	3.64621E-17	3	3	3	12.924	1
59	6.946E-05	0.00086	4.74E-05	0.00065	4.349071	4.349071	1.60829E-09	7.47819E-10	1.2933E-18	2.79617E-19	3	3	3	12.924	1
60	1.871E-05	0.00014	2.09E-05	0.00021	4.254772	4.254772	1.16656E-10	1.45633E-10	6.80428E-21	1.06044E-20	3	3	3	12.924	1
61	4.686E-05	0.0005	4E-05	0.00051	0.425752	0.425752	7.32069E-10	5.3362E-10	2.67962E-19	1.42375E-19	3	3	3	12.924	1

Figure A2.3 Automated discriminating ion output example

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REFERENCES

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III. Intra- and Inter-Month Statistical Comparison of Fluoroisobutyryl and Fluorobutyryl Fentanyl Isomers

3.1 Mass Spectra of Fentanyl Isomers

3.1.1 Fluoroisobutyryl Fentanyl Isomers

Fluoroisobutyryl fentanyl (FIBF) is an analog of fentanyl that includes modifications of the core fentanyl structure in both the amide group and aniline ring regions. Within the amide group, an isobutyryl group is added to the core structure and a fluorine group is positioned on the aromatic ring. Due to the three possible positions for substitution around the aniline ring, there are three positional isomers of FIBF: *ortho* (*o*)-FIBF, *meta* (*m*)-FIBF, and *para* (*p*)-FIBF. Structures of the isomers are shown in Figure 3.1 A-C, highlighting the substitutions on the amide group and around the aniline ring.

Representative normalized spectra of *o*-FIBF, *m*-FIBF, and *p*-FIBF are shown in Figure 3.1 D-F. The molecular ion of all three of the isomers is at mass-to-charge (*m/z*) 369; however, it was not visible in the spectra. Visual inspection of the ion abundances relative to the base peak (*m/z* 277) demonstrate the spectral similarities among the isomers. Comparable relative abundances of ions such as *m/z* 43 and *m/z* 207 were observed across the spectra. The dominant ion of *m/z* 207 is known to be a common background ion in gas chromatography-mass spectrometry (GC-MS). However, in the FIBF isomers, *m/z* 207 is known to be chemically relevant and important for the identification of these compounds.[1] After close inspection, small differences were observed in the relative ion abundances of *m/z* 71 and *m/z* 164. In *o*-FIBF, *m/z* 71 was present with an abundance less than 20% relative to the base peak whereas, in *m*- and *p*-FIBF, this ion was present at a relative abundance greater than 20%. In contrast, the ion at *m/z*

164 was present at higher relative abundance in *o*-FIBF compared to *m*- and *p*-FIBF (relative abundance of 60% compared to 40% and 45%, respectively).

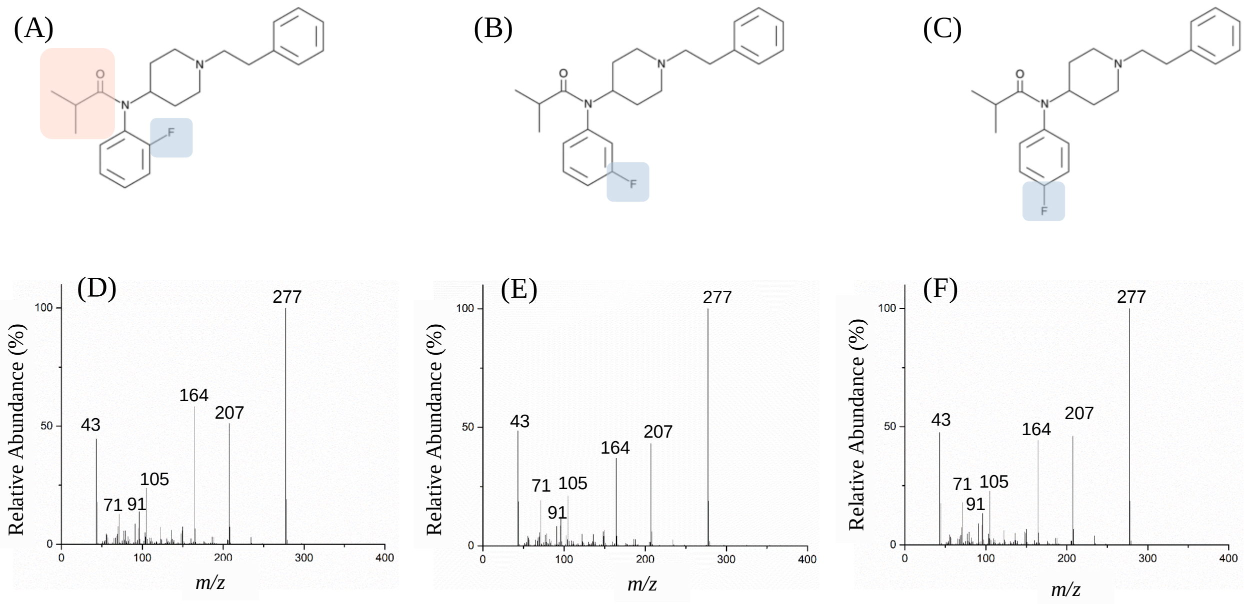

Figure 3.1 Representative mass spectra of (A) *ortho*-fluoroisobutyryl fentanyl (*o*-FIBF), (B) *meta*-fluoroisobutyryl fentanyl (*m*-FIBF), and (C) *para*-fluoroisobutyryl fentanyl (*p*-FIBF)

In addition to visually similar spectra, pairwise comparisons of the spectra of the three positional isomers of FIBF had high PPMC coefficients, indicating strong correlation. The mean PPMC coefficient among replicates collected over a few days for comparison of *o*-FIBF spectra was 0.9999 ± 0.0001. For *m*-FIBF, the mean PPMC coefficient among five injection replicates collected in Month 1 was 0.9992 ± 0.0007, while for *p*-FIBF, the mean PPMC coefficient was 0.9994 ± 0.0005. Comparing the correlation between different isomers, the PPMC coefficient between *o*-FIBF and *m*-FIBF was 0.9827 ± 0.0009. The comparison between *o*-FIBF and *p*-FIBF had a PPMC of 0.9933 ± 0.0005. Finally, the PPMC coefficient of the comparison between *m*-FIBF and *p*-FIBF was 0.9959 ± 0.0014. The strong correlation among the spectra of the isomers demonstrates the level of spectral similarity, which makes it difficult to differentiate one isomer from another when relying solely on visual inspection of spectra.

The spectral data were also searched against the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health (NIST/EPA/NIH) Mass Spectral Library, using the probability-based matching (PBM) algorithm in the Agilent software. For the FIBF isomers, for five replicates of *o*-FIBF, the top hit was always *p*-FBF, which is a different structural isomer that will be described in the next section. The match quality was 81 for four of the five replicates and 90 for one. The second hit for the five replicates of *o*-FIBF was *o*-FBF, again a structural isomer, with a match quality of 70. For the five replicates of *m*-FIBF, four resulted in top hits of *p*-FBF and one had a top hit of *o*-FBF with a match quality ranging from 62 to 83. Finally, for the five replicates of *p*-FIBF, four of the top hits were labeled FIBF without any positional isomer information and one of the top hits was *p*-FBF. The match qualities were either 90 or 93. Although there is no confirmation, it is believed that the mass spectra of *o*-FIBF and *m*-FIBF reference standards are not included in the library and FIBF is considered to be the *para* isomer. However, it is interesting that the top hits for both *o*-FIBF and *m*-FIBF were isomers of FBF instead of FIBF.

3.1.2 Fluorobutyryl Fentanyl Isomers

Fluorobutyryl fentanyl (FBF) is another analog of fentanyl with modifications to the core structure in the amide group and aniline ring regions. In this case, FBF contains the same fluorine substitutions on the aniline ring as FIBF, but differs from FIBF in the presence of a butyryl, rather than an isobutyryl, group on the amide group. Similar to FIBF, there are three positional isomers of FBF according to the fluorine substitution: *o*-FBF, *m*-FBF, and *p*-FBF. The structures of *o*-, *m*-, and *p*-FBF are shown in Figure 3.2 A-C, highlighting the butyryl substitution on the amide group and the fluorine substitutions around the aniline ring.

Representative spectra of *o*-FBF, *m*-FBF, and *p*-FBF are shown in Figure 3.2 D-F. Once again, the spectra of the three positional isomers were very similar, with comparable ion abundances. The relative abundances of ions such as *m/z* 43 and *m/z* 105 cannot be visually distinguished. Upon closer inspection, the relative abundance of *m/z* 164 varied among the three isomers, ranging from 70% relative abundance in *o*-FBF to relative abundance of 40% and 50% in *m*-FBF and *p*-FBF, respectively.

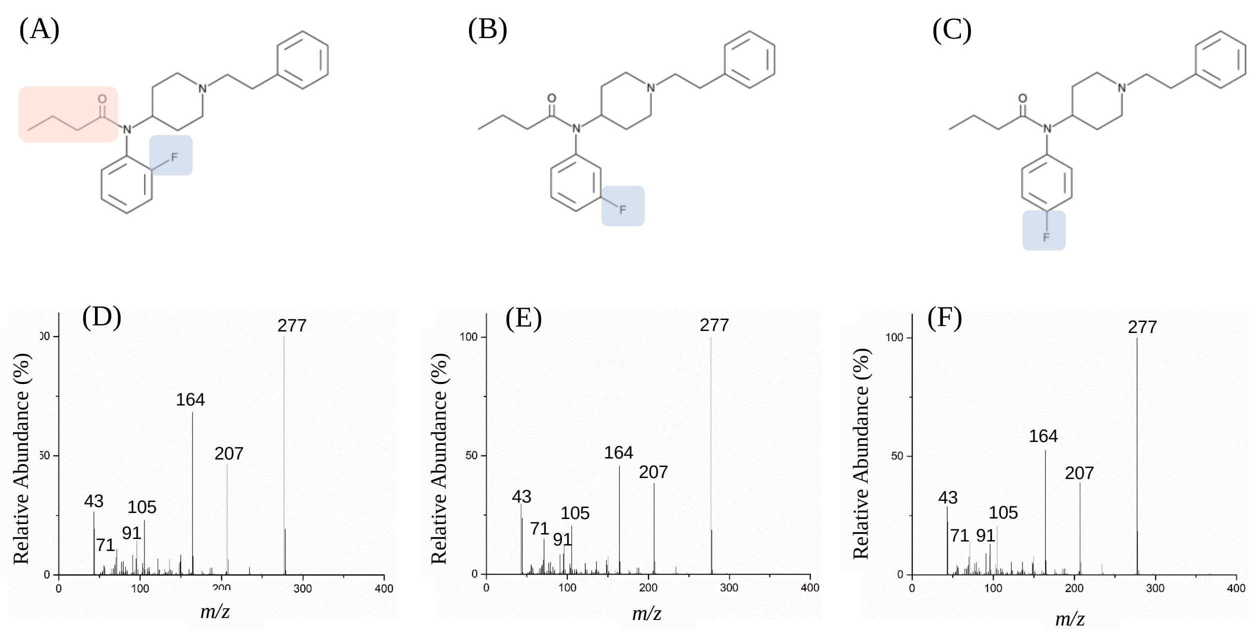

Figure 3.2 Representative mass spectra of (A) *ortho*-fluorobutyryl fentanyl, (B) *meta*-fluorobutyryl fentanyl, and (C) *para*-fluorobutyryl fentanyl

Not only were the spectra of the FBF positional isomers visually similar, but the PPMC coefficients once again prove high correlation between the spectra. The mean PPMC coefficients for comparison of corresponding isomers were 0.9997 ± 0.0003, 0.9997 ± 0.0002, and 0.9997 ± 0.0002, for injection replicates of *o*-FBF, *m*-FBF, and *p*-FBF respectively. Comparing the correlation between different isomers collected in Month 1, the PPMC coefficient between *o*-FBF and *m*-FBF was 0.9849 ± 0.0012. The comparison between *o*-FBF and *p*-FBF had a PPMC of 0.9924 ± 0.0009. And finally, the PPMC coefficient of the comparison between *m*-FBF and *p*-FBF was 0.9981 ± 0.0005. While all of the PPMC coefficients demonstrate strong correlation, it is important to note the high similarity of the spectra from different isomers.

Spectra of the FBF isomers were also compared to the NIST/EPA/NIH Mass Spectral Library using the PBM algorithm. The correct isomer was the top hit for the five *o*-FBF replicates, with match qualities of either 93 or 95. For the five replicates of *m*-FBF, four of the top hits were *p*-FBF and one of the top hits was *o*-FBF, all with a match quality of 90. Finally, for the five replicates of *p*-FBF, the top hit was always correct with a match quality ranging from 87 to 93. It should be noted in the case of the FBF isomers that, although not confirmed, it is believed that the mass spectrum of *m*-FBF reference is not included in the library and is believed to be the cause of the incorrect hits for *m*-FBF.

When visually comparing representative spectra of the FIBF isomers and the FBF isomers (Figures 3.1 and 3.2 D-F), small differences were observed between the relative abundances of *m/z* 43, 164, and 207. However, the six spectra are still very visually similar as all six compounds are isomers of each other. The PPMC coefficients show strong correlation among the isomers, with mean coefficients ranging from 0.9466 to 0.9882 (Appendix Table A3.1).

3.2 Intra-Month Comparisons of FIBF and FBF Spectra to FIBF Reference Spectra

While comparisons were performed for all pairwise combinations of the six isomers, this chapter focuses on comparison of FIBF and FBF spectra to the FIBF reference spectra. Initially, spectra of isomers collected each month were compared to the corresponding FIBF reference spectra collected the same month.

3.2.1 Month 1 FIBF and FBF Spectra Compared to the Month 1 FIBF Reference Spectra

The FIBF and FBF comparison spectra collected in Month 1 were compared to the corresponding Month 1 FIBF reference spectra. In Month 1, two collections of FIBF comparison spectra samples were analyzed – Month 1A and Month 1B – to be compared to the same Month 1 reference spectra. These comparisons were used to evaluate the effect of variation in the spectral intensities on the ability to properly associate and discriminate the spectra. The two collections were only made for the FIBF isomers in Month 1.

Corresponding spectra of *o*-FIBF were statistically associated at the 99.9% confidence level (Table 3.1). For these comparisons, spectrum A and spectrum B (from Month 1A and 1B, respectively) were correctly associated to the *o*-FIBF reference spectra with a minimum random-match probability (P_{min}) of 4.749×10^{-55} and a maximum random-match probability (P_{max}) of 4.157×10^{-24} calculated across the scan range *m/z* 40-400. Similarly, corresponding spectra of *m*-FIBF were statistically associated at the 99.9% confidence level (Table 3.1). Both *m*-FIBF comparison spectrum A and B were associated to the corresponding *m*-FIBF reference spectra at the 99.9% confidence level with $P_{\text{min}} = 2.474 \times 10^{-52}$ and $P_{\text{max}} = 1.185 \times 10^{-24}$. These low random-match probabilities demonstrate how unlikely it was that the mass spectral patterns occurred by random chance alone.

Table 3.1 Comparison of two FIBF comparison spectrum data collections in Month 1 (A and B) to Month 1 FIBF reference mass spectra at the 99.9% confidence level

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Month 1 A	Month 1 B	Month 1 A	Month 1 B
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	9	13	43, 71, 90, 102, 118, 144, 149, 164, 165	70, 71, 90, 102, 110, 111, 122, 130, 144, 149, 164, 165, 185
	p-FIBF	9	10	43, 71, 90, 102, 118, 144, 149, 164, 165	71, 84, 90, 111, 112, 130, 143, 144, 164, 165
***m*-FIBF**	*o*-FIBF	7	8	43, 71, 90, 111, 148, 164, 165	43, 71, 90, 95, 118, 148, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	1	2	234	84, 234
***p*-FIBF**	*o*-FIBF	3	2	71, 164, 234	71, 164
	m-FIBF	2	4	164, 234	70, 110, 164, 234
	p-FIBF	0	1	–	366

For *p*-FIBF, correct association was observed for comparisons between the Month 1A spectra and the *p*-FIBF reference spectra at the 99.9% confidence level, with $P_{min} = 4.706 \times 10^{-54}$ and $P_{max} = 3.274 \times 10^{-25}$. However, the Month 1B spectra were incorrectly discriminated from the *p*-FIBF reference spectra with one ion (*m/z* 366) responsible for discrimination. For this ion, the t_{calc} value of 14.218 was greater than the t_{crit} value of 12.924, causing the rejection of the null hypothesis. The chemical relevance of *m/z* 366 is not known, but the ion does not appear as a common discriminating ion in other comparisons.

In terms of discrimination, the Month 1 FIBF comparison spectra were correctly discriminated from the other FIBF reference spectra in all cases (Table 3.1). There was a similar number of discriminating ions between the spectrum A and spectrum B comparisons in Month 1, however the identities of those ions varied between each collection.

The *m*- and *p*-FIBF comparison spectra were discriminated from the *o*-FIBF reference spectra at the 99.9% confidence level (Table 3.1). The *m*-FIBF to *o*-FIBF comparison resulted in 9 discriminating ions for the Month 1A collection and 13 discriminating ions for the Month 1B collection. Common discriminating ions between the spectra of *m*- and *o*-FIBF for both collections included *m/z* 71, 90, 102, 144, 149, 164, and 165. The *p*-FIBF to *o*-FIBF reference spectra comparison resulted in 9 discriminating ions for the Month 1A spectrum and 10 discriminating ions for the Month 1B spectrum. The month 1A and 1B comparisons of *p*-FIBF to *o*-FIBF resulted in common ions between both collections such as *m/z* 71, 90, 144, 164, and 165.

Discrimination of the *o*- and *p*-FIBF comparison spectra from the *m*-FIBF reference spectra was also possible at the 99.9% confidence level (Table 3.1). For *o*-FIBF compared to the *m*-FIBF reference, 7 and 8 ions were responsible for discrimination in the Month 1A spectrum and Month 1B spectrum, respectively. Common discriminating ions between *o*- and *m*-FIBF for both collections included *m/z* 43, 71, 90, 148, 164, and 165. For *p*-FIBF comparison spectra and the *m*-FIBF reference, discrimination was also observed at the 99.9% confidence level, with 1 to 2 discriminating ions observed (Table 3.1). For Month 1A, the discriminating ion was *m/z* 234, whereas for Month 1B, *m/z* 234 and *m/z* 84 were discriminating ions.

The *o*- and *m*-FIBF spectra in both Month 1A and Month 1B were correctly discriminated from the *p*-FIBF reference spectra at the 99.9% confidence level (Table 3.1). For the *o*-FIBF to *p*-FIBF comparison, 3 and 2 discriminating ions were observed for the Month 1A and Month 1B collections, respectively. For these comparisons, the common ions were *m/z* 71 and *m/z* 164, both of which were previously observed for the comparison of *p*-FIBF to the *o*-FIBF reference spectra (Table 3.1). For the comparison of *m*-FIBF and the *p*-FIBF reference, there were 2 and 4 discriminating ions for Month 1A and Month 1B, respectively. The common ions responsible for discrimination were *m/z* 164 and *m/z* 234, where the latter was also observed in the comparison between *p*-FIBF comparison spectrum and *m*-FIBF reference spectra.

The relative abundances of these common discriminating ions relating to all previously discussed comparisons ranged from as low as 0.3% up to 67%, but a majority of the ions had a relative abundance less than 5% of the base peak. This reiterates the need for a statistical comparison of the isomers beyond a visual comparison, as those differences in abundances become more difficult to observe at lower relative abundance. In addition, this demonstrates the need to include the entire mass spectrum instead of only the most abundant ions, as those with lower relative abundance are important for discrimination. Referring back to differences in ion abundances that were observed visually in the spectra of FIBF isomers in Figure 3.1, *m/z* 71 and *m/z* 164 were two ions in which the differences in relative abundance between the *o*-FIBF isomer and the other two FIBF isomers were apparent. Those ions were also determined to be common ions responsible for the discrimination of *o*-FIBF from the other two isomers. Although two of the ions responsible for discrimination could be observed through visual inspection of the spectra, more ions were apparent with the statistical comparison method, making the discrimination more robust and adding statistical confidence to the differentiation.

The statistical comparison method was also used to compare spectra of the structural isomers FIBF and FBF. In these cases, the FIBF positional isomers were used as the reference spectra and the FBF spectra collected in Month 1 were used as the comparison spectra. Spectra for the FBF isomers were only collected once during Month 1, corresponding to Month 1B.

All FBF comparison spectra were correctly discriminated from the FIBF reference spectra at the 99.9% confidence level with a range of 3-19 ions responsible for discrimination (Table 3.2). In all nine comparisons, *m/z* 43 was present as a discriminating ion. This ion was also highlighted in the visual assessment of the mass spectra (Figures 3.1 and 3.2), in which *m/z* 43 was present at higher abundance in FIBF (50% relative abundance) compared to FBF (28% relative abundance).

When comparing *o*-FBF to all FIBF reference spectra, *m/z* 43, 113, and 164 were present as discriminating ions. In the comparisons between *o*-FBF and the *m*- and *p*-FIBF reference spectra, eleven common discriminating ions were observed (Table 3.2). The comparison of *m*-FBF comparison spectra to the three FIBF reference spectra resulted in one common ion, *m/z* 43. And the comparison between *m*-FBF and the *p*-FIBF reference spectra resulted in one especially interesting discriminating ion (*m/z* 366), which was observed previously in the *p*-FIBF to *p*-FIBF comparison in Month 1B (Table 3.1) and which resulted in an incorrect discrimination (false negative). Lastly, the comparison of *p*-FBF comparison spectra to the FIBF reference spectra resulted in the common discriminating ion *m/z* 43.

Table 3.2 Comparison of Month 1 FBF comparison spectra to corresponding FIBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FIBF**	*o*-FBF	3	43, 113, 164
	m-FBF	12	43, 44, 90, 105, 111, 122, 130, 144, 149, 164, 190, 208
	p-FBF	11	43, 44, 84, 90, 122, 130, 144, 176, 185, 208, 234
***m*-FIBF**	*o*-FBF	14	43, 71, 90, 102, 110, 112, 113, 118, 143, 144, 149, 164, 165, 176
	m-FBF	3	43, 71, 113
	p-FBF	7	43, 71, 84, 164, 176, 234, 235
***p*-FIBF**	*o*-FBF	19	43, 71, 72, 90, 95, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 159, 164, 165, 166
	m-FBF	6	43, 44, 71, 122, 149, 366
	p-FBF	3	43, 44, 71

3.2.2 Month 2 FIBF and FBF Spectra Compared to Month 2 FIBF Reference Spectra

The FIBF and FBF comparison spectra collected in Month 2 were statistically compared to the corresponding Month 2 FIBF reference spectra (Table 3.3). Corresponding spectra of *o*-FIBF, *m*-FIBF, and *p*-FIBF were all statistically associated at the 99.9% confidence level in Month 2 (Table 3.3). The *o*-FIBF comparison spectrum and reference spectra were correctly associated with $P_{\min} = 5.731\text{x}10^{-56}$ and $P_{\max} = 5.172\text{x}10^{-24}$. For *m*-FIBF, the comparison spectrum and reference spectra were associated with $P_{\min} = 2.801\text{x}10^{-55}$ and $P_{\max} = 1.680\text{x}10^{-23}$.

Lastly, the comparison spectrum of *p*-FIBF was associated to the corresponding reference spectra with $P_{\min} = 3.160 \times 10^{-55}$ and $P_{\max} = 3.330 \times 10^{-24}$. In all three cases, the probability that the mass spectral fragmentation patterns occurred by random chance alone was very small.

Table 3.3 Comparison of Month 2 FIBF comparison spectra and corresponding reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FIBF**	*o*-FIBF	0	–
	m-FIBF	8	43, 44, 71, 90, 102, 111, 148, 164
	p-FIBF	5	71, 111, 112, 164, 234
***m*-FIBF**	*o*-FIBF	7	71, 90, 102, 118, 149, 164, 165
	m-FIBF	0	–
	p-FIBF	1	234
***p*-FIBF**	*o*-FIBF	5	71, 90, 130, 143, 164
	m-FIBF	5	43, 44, 71, 84, 234
	p-FIBF	0	–

In terms of discrimination, the Month 2 comparison FIBF spectra were correctly discriminated from the FIBF reference spectra at the 99.9% confidence level in all cases (Table 3.3). The *m*- and *p*-FIBF comparison spectra were discriminated from the *o*-FIBF reference spectra with 8 and 5 ions responsible for discrimination, respectively. Common discriminating ions resulting from these two comparisons to *o*-FIBF included *m/z* 71, 111, and 164.

Discrimination of the *o*- and *p*-FIBF comparison spectra from the *m*-FIBF reference spectra was possible at the 99.9% confidence level with 7 and 1 ions responsible for discrimination, respectively. There were no common discriminating ions when using *m*-FIBF spectra as the reference to compare to the other two isomers.

When comparing the *o*- and *m*-FIBF comparison spectra to the *p*-FIBF reference spectra, discrimination was possible at the 99.9% confidence level for Month 2, with 5 ions responsible for both comparisons. Once again, there were no common discriminating ions when using *p*-FIBF as the reference spectra to compare to the other positional isomers.

While there were no common discriminating ions when using *m*- and *p*-FIBF as the reference spectra in comparisons, there were common discriminating ions observed when looking at each isomer's comparison spectra to the different reference spectra. When using *o*-FIBF as the comparison spectrum to the *m*- and *p*-FIBF reference spectra, *m/z* 71 90, and 164 were common ions in the two comparisons. When using *m*-FIBF as the comparison spectrum to compare to the *o*- and *p*-FIBF reference spectra, *m/z* 43, 44, and 71 were common ions observed in the two comparisons. Finally, when using *p*-FIBF as the comparison spectrum to the *o*- and *m*-FIBF reference spectra, *m/z* 234 was a common ion observed in the two comparisons.

The statistical comparison method was also used to compare spectra of the structural isomers FIBF and FBF in Month 2. The FIBF spectra remained the reference spectra and the FBF spectra were used as the comparison spectra. All comparisons were performed at the 99.9% confidence level and discrimination was possible for all nine comparisons with a range of 2-17 discriminating ions identified (Table 3.4).

Table 3.4 Comparison of Month 2 FBF comparison spectra to corresponding FIBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FIBF**	*o*-FBF	4	44, 111, 113, 164
	m-FBF	7	44, 71, 95, 111, 118, 122, 148
	p-FBF	7	43, 44, 90, 102, 118, 176, 234
***m*-FIBF**	*o*-FBF	13	43, 90, 102, 110, 112, 113, 136, 143, 144, 149, 164, 165
	m-FBF	3	43, 44, 164
	p-FBF	5	43, 71, 164, 176, 234
***p*-FIBF**	*o*-FBF	17	43, 44, 71, 90, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 150, 164, 165
	m-FBF	2	43, 44
	p-FBF	3	43, 44, 71

When using *o*-FBF as the comparison spectrum, common ions responsible for discrimination from the FIBF isomers included *m/z* 113 and *m/z* 164. These two ions were also commonly responsible for discrimination in the Month 1 comparisons. When comparing the *m*-FBF comparison spectrum to the FIBF reference spectra, *m/z* 44 was a discriminating ion in all three comparisons, where the relative abundance was higher in the *m*-FBF comparison spectrum (25%) than in the FIBF reference spectra (16%). Finally, the common discriminating ion observed when comparing the *p*-FBF comparison spectrum to the FIBF reference spectra was *m/z* 43, which again, was always present at higher abundance in the FIBF reference spectra than in any FBF comparison spectrum.

3.2.3 Month 3 FIBF and FBF Spectra Compared to Month 3 FIBF Reference Spectra

The FIBF and FBF comparison spectra in Month 3 were compared to the corresponding Month 3 FIBF reference spectra. Results from the comparisons of the FIBF comparison spectra are summarized in Table 3.5. In terms of association, all corresponding spectra of *o*-, *m*-, and *p*-FIBF were associated at the 99.9% confidence level. In Month 3, the *o*-FIBF comparison spectrum and reference spectra were correctly associated with $P_{\min} = 6.174\text{x}10^{-57}$ and $P_{\max} = 9.038\text{x}10^{-22}$. For *m*-FIBF, the comparison spectrum and reference spectra were associated with $P_{\min} = 3.214\text{x}10^{-56}$ and $P_{\max} = 2.104\text{x}10^{-23}$. Finally, the comparison spectrum of *p*-FIBF was associated to the corresponding reference spectra with $P_{\min} = 6.244\text{x}10^{-56}$ and $P_{\max} = 5.345\text{x}10^{-21}$. Once again, the low random-match probabilities indicate the very low probability that these mass spectral fragmentation patterns occurred by random chance alone. For each isomer, the random-match probabilities were slightly different in Months 1, 2, and 3. This demonstrates the slight variabilities in the spectral collections over the individual months.

In terms of discrimination, the FIBF comparison spectra collected in Month 3 were discriminated from the FIBF reference spectra at the 99.9% confidence level in most cases. The comparisons between the *m*- and *p*-FIBF comparison spectra and the *o*-FIBF reference spectra resulted in 5 and 3 ions responsible for discrimination, respectively. Common discriminating ions resulting from the comparisons to reference *o*-FIBF included *m/z* 71 and *m/z* 164.

The *o*-FIBF comparison spectrum was discriminated from the *m*-FIBF reference spectra at the 99.9% confidence level, resulting in 7 discriminating ions (Table 3.5). However, the *p*-FIBF comparison spectrum was not discriminated from the *m*-FIBF reference spectra; therefore, there were no discriminating ions at the 99.9% confidence level, resulting in a false positive. In previous months, *m/z* 234 was a common discriminating ion. In this comparison, the abundances at that *m/z* value did not fail the *t*-test as t_{calc} was less than t_{crit} (12.364 versus 12.924). As only the *o*-FIBF comparison spectrum was discriminated from the *m*-FIBF reference spectra, there were no common discriminating ions observed for the comparisons to *m*-FIBF.

The comparisons of *o*- and *m*-FIBF comparison spectra to the *p*-FIBF reference spectrum resulted in 5 and 2 discriminating ions, respectively. The common discriminating ion resulting from the two comparisons to the *p*-FIBF reference spectra was *m/z* 164.

Table 3.5 Comparison of Month 3 FIBF comparison spectra and corresponding reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FIBF**	*o*-FIBF	0	–
	m-FIBF	5	71, 90, 102, 164, 165
	p-FIBF	3	71, 95, 164
***m*-FIBF**	*o*-FIBF	7	71, 95, 102, 118, 149, 164, 165
	m-FIBF	0	–
	p-FIBF	0	–
***p*-FIBF**	*o*-FIBF	5	71, 90, 95, 144, 164
	m-FIBF	2	164, 234
	p-FIBF	0	–

Common discriminating ions were also present when analyzing the trends in the comparison spectra separately. The comparisons between the *o*-FIBF as the comparison spectrum and the *m*- and *p*-FIBF reference spectra resulted in common discriminating ions including *m/z* 71 and *m/z* 164. There was only one successful discrimination involving *m*-FIBF;

therefore, there were no common discriminating ions using that comparison spectrum. When using the *p*-FIBF comparison spectrum to compare to the *o*- and *m*-FIBF reference spectra, there was a common discriminating ion at *m/z* 164.

The statistical comparison method was also used to compare the comparison spectra of FBF isomers to the corresponding FIBF reference spectra collected in Month 3. All nine comparisons were performed at the 99.9% confidence level. Discrimination was possible for all comparisons with a range of 2 to 14 discriminating ions (Table 3.6).

When using *o*-FBF as the comparison spectrum to each of the three FIBF reference spectra, common discriminating ions include *m/z* 43 and *m/z* 164. When using *m*-FBF as the comparison spectrum to the FIBF reference spectra, comparisons resulted in the common discriminating ions of *m/z* 43 and *m/z* 44. Finally, using *p*-FBF as the comparison spectrum resulted only in the common discriminating ion at *m/z* 43.

Table 3.6 Comparison of Month 3 FBF comparison spectra to corresponding FIBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FIBF**	*o*-FBF	2	43, 164
	m-FBF	8	43, 44, 71, 93, 95, 122, 148, 164
	p-FBF	3	43, 44, 234
***m*-FIBF**	*o*-FBF	6	43, 90, 102, 118, 164, 165
	m-FBF	4	43, 44, 93, 164
	p-FBF	3	43, 164, 234

Table 3.6 (cont'd.)

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***p*-FIBF**	*o*-FBF	14	43, 71, 90, 95, 102, 112, 118, 124, 130, 136, 143, 144, 164, 165
	m-FBF	2	43, 44
	p-FBF	4	43, 44, 111, 164

3.2.4 Trends in the Month 1 – 3 Intra-Month Comparisons to FIBF Reference Spectra

As the statistical comparison method was used to compare FIBF reference spectra to both FIBF and FBF comparison spectra across three months, the results for Month 1B, Month 2, and Month 3 comparisons were further investigated to determine the presence of trends in common discriminating ions.

For comparisons of the FIBF spectra (Tables 3.1, 3.3, and 3.5), a higher number of discriminating ions was observed when comparing the *o*-FIBF isomer to the other two FIBF positional isomers. This was due to the higher relative abundance of many ions in the *ortho*-isomer, which may be explained due to the *ortho*-effect.[2] This phenomenon occurs because of an increase (or sometimes a decrease) in the abundance of certain ions in the mass spectrum of an *ortho* isomer due to the placement of the substituent on the ring. In the *ortho*-position, there are often alternative fragmentation pathways that exist compared to *meta*- and *para*-positions. This results in an increase in abundance of the ion in the *ortho* isomer which, in this work, led to a statistical difference at that *m/z* value and an increase in the number of discriminating ions. The substituent in the case of both FIBF and FBF is fluorine which is electron-withdrawing, but *ortho*-/*para*- directing.[2] One example is *m/z* 164, which always has a higher relative abundance in the *o*-FIBF spectra than the spectra of the other two positional isomers. This ion (*m/z* 164) is thought to occur *via* two different fragmentation pathways (from *m/z* 234 and *m/z* 207) and includes the fluorine substituent in the fragment (Figure 3.3).[1] The increase in abundance of the ion in the *o*-FIBF spectrum is likely due to the *ortho*-effect.

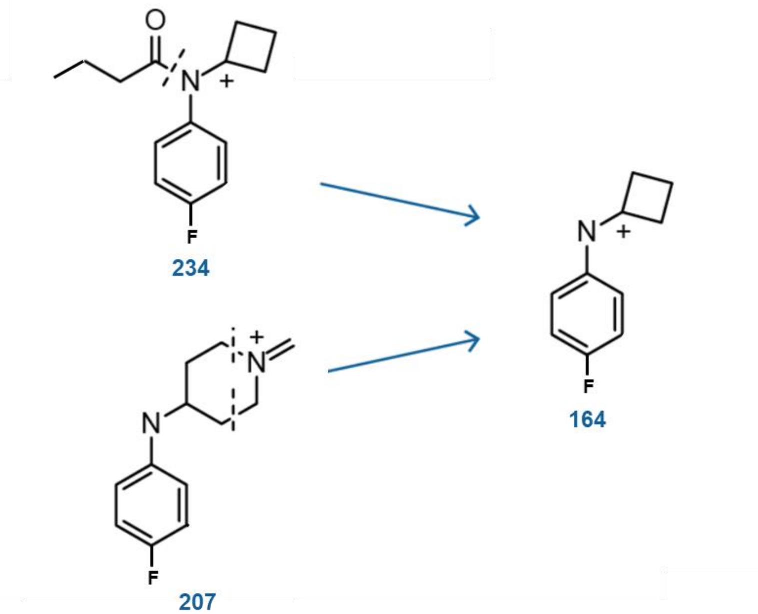

Figure 3.3 The proposed structure and formation pathways of *m/z* 164.[1]

Another trend that was observed in the FIBF comparisons was that the number of discriminating ions for each of the six discrimination comparisons varied only slightly across the three-month study. Although the number of discriminating ions was relatively consistent, the identity of the ions was not always consistent. For example, in the comparison of the *o*-FIBF comparison spectrum and the *m*-FIBF reference spectra, there were 8, 7, and 7 discriminating ions in Months 1, 2, and 3, respectively. However, only four ions (*m/z* 71, 118, 164, and 165) were common discriminating ions across all three months for this comparison. With the three months of data collected, ions that were reliable for the discrimination of the pairs of isomers were able to be identified.

For the discrimination of the *o*- and *m*-FIBF pair of isomers (*m*-FIBF as the comparison to the *o*-FIBF reference and *o*-FIBF as the comparison to the *m*-FIBF reference), *m/z* 71, 90, 102, and 164 were identified as discriminating ions in all three months of comparisons. Because these ions were common for all three months, they have been identified as four ions that would reliably distinguish the *ortho* and *meta* isomers of FIBF. For *m/z* 71, the relative abundance was higher in the *m*-FIBF spectra than in the *o*-FIBF spectra at an average of 25% and 18%, respectively. For the remaining common ions of *m/z* 90, 102, and 164, the relative abundances were all higher in the *o*-FIBF spectra at averages of 1%, 2%, and 65%, respectively. These abundances were higher potentially due to the *ortho*-effect phenomenon.

For the discrimination between the pair of *o*- and *p*-FIBF isomers (*p*-FIBF as the comparison to the *o*-FIBF reference and *o*-FIBF as the comparison to the *p*-FIBF reference), *m/z* 71 and 164 were identified as discriminating ions in all three months of comparisons. Because these ions were observed in all three months, they have been identified as two ions that would reliably distinguish between *o*- and *p*-FIBF. The relative abundance of *m/z* 71 was higher in the *p*-FIBF spectra than in the *o*-FIBF spectra at averages of 22% and 18%, respectively. For *m/z* 164, the relative abundance was higher in the *o*-FIBF spectra than in the *p*-FIBF spectra, with average abundances of 65% and 42%, respectively.

For the discrimination between the pair of *p*- and *m*-FIBF isomers (*m*-FIBF as the comparison to the *p*-FIBF reference and *p*-FIBF as the comparison to the *m*-FIBF reference), one ion, *m/z* 234, was identified as a common discriminating ion in all three months of comparisons. Because this ion was observed in all three months, it has been identified as one that would reliably distinguish between the two isomers of *p*- and *m*-FIBF. The relative abundance of *m/z* 234 was higher in the *p*-FIBF spectra than in the *m*-FIBF spectra, with an average relative abundance of 4% and 3%, respectively. While there is only a 1% difference in abundance here, this was a statistically significant difference that enabled the distinction of the two isomers.

For the trends in the comparisons between the FBF comparison spectra and the FIBF reference spectra, overall, there was a higher number of discriminating ions present, as expected, ranging from 3 – 19 ions for the comparisons (Tables 3.2, 3.4, and 3.6). Common ions that resulted in discrimination of all of the FBF comparison spectra from the *o*-FIBF reference spectra in all three months included *m/z* 43, 44, 111, 122, and 164. For *m/z* 43, 111, and 122, the average relative abundance of the *o*-FIBF reference spectra was higher than the relative abundance of each of the FBF comparison spectra. For example, the relative abundance of *m/z* 111 was 6% in the *o*-FIBF reference spectra and 4% in the FBF comparison spectra. The abundance of *m/z* 122 was 8% in the *o*-FIBF reference spectra and 6% in the FBF comparison spectra. For *m/z* 44 and *m/z* 164, the opposite was true. The relative abundance of *m/z* 44 was lower at 21% in the *o*-FIBF reference spectra and higher at 35% in the FBF comparison spectra.

Common ions that resulted in discrimination of the FBF comparison spectra from the *m*-FIBF reference spectra across the three months included *m/z* 43, 71, 164, and 234. For *m/z* 43 and *m/z* 71, the relative abundances were higher in the FIBF reference spectra with averages of 45% and 20%, respectively. However; the relative abundances of *m/z* 164 (68%) and *m/z* 234 (5%) were higher in the FBF comparison spectra.

Finally, when comparing the FBF comparison spectra to the *p*-FIBF reference spectra, only *m/z* 43 was identified as a common discriminating ion observed in all three months. Following the same trends as the comparisons to the other two FIBF isomers as reference spectra, the relative abundance of *m/z* 43 was higher in the *p*-FIBF spectra than in the FBF comparison spectra.

All nine discrimination comparisons between FBF and FIBF included *m/z* 43 as a discriminating ion. For each of the nine comparisons, the average relative abundance of this ion was higher for the FIBF reference spectra than the FBF comparison spectra, which has been demonstrated repeatedly. Referring back to the spectra of FIBF and FBF isomers in Figures 3.1 and 3.2, there was a visual difference in the abundance of *m/z* 43 between the FIBF and FBF spectra. Some of the common ions observed in the FBF to FIBF reference spectra comparisons were also observed in the FIBF to FIBF comparisons (*m/z* 43, 71, 90, 164, and 234). However, there were ions that were not observed during the FIBF to FIBF comparisons including low abundance ions (below 5% of the base peak) such as *m/z* 102, 111, 118, 122, 124, 130, 136, 143, and 165. The relative abundances of these ions ranged from 0.2% to 10%.

Comparisons were also made using FBF isomers as the reference spectra to the FBF and FIBF isomers as comparison spectra. The results of these comparisons are shown in Appendix Tables A3.2 – A3.7. The overall trends for these comparisons were similar to the trends observed in the FIBF comparisons. However, there were some differences in the identities of the ions responsible for discrimination in the FBF to FBF comparisons and the FIBF to FBF comparisons.

3.4 Inter-Month Comparisons of FIBF and FBF Spectra to FIBF Reference Spectra

Because the electron multiplier response between Month 1 and Month 2 was not statistically different, comparisons of spectra collected between these two months were compared. Spectra collected in Month 1 were retained as the reference to which spectra of FIBF and FBF collected in Month 2 were compared.

For comparisons of FIBF comparison spectra collected in Month 2 to the FIBF reference spectra collected in Month 1, no association was observed, resulting in three instances of incorrect discriminations (false negatives) (Table 3.7). In addition, the numbers of discriminating ions between different positional isomers was higher than previously observed during each of the intra-month comparisons, with up to 33 ions responsible for discrimination. The identities of some of the ions were the same as those observed in the intra-month comparisons including *m/z* 43, 44, 70, 71, 91, 102, 164, and 234. However, many of the ions identified as being discriminatory had not been observed previously for these comparisons, for example, *m/z* 55, 58, 65, 67, and 77. The relative abundances of these ions change between Month 1 and Month 2, causing differences between the isomers that were not observed previously.

Table 3.7 Inter-month comparison of Month 1 FIBF as reference spectra and Month 2 FIBF as comparison spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	m/z Values of Discriminating Ions
o-FIBF	o-FIBF	2	43, 44
	m-FIBF	14	43, 44, 55, 58, 71, 72, 91, 95, 102, 105, 118, 148, 164, 190
	p-FIBF	9	43, 44, 71, 72, 91, 95, 148, 164, 234
m-FIBF	o-FIBF	21	70, 71, 77, 90, 96, 102, 105, 109, 110, 111, 112, 118, 122, 130, 136, 143, 144, 149, 159, 164, 165
	m-FIBF	9	43, 44, 55, 68, 70, 71, 91, 96, 105
	p-FIBF	6	70, 96, 109, 149, 164, 234

Table 3.7 (cont'd.)

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***p*-FIBF**	*o*-FBF	33	43, 44, 51, 54, 55, 56, 65, 67, 68, 70, 76, 77, 79, 84, 94, 96, 98, 105, 110, 111, 112, 116, 117, 122, 124, 128, 130, 131, 136, 144, 164, 165, 185
	m-FBF	20	43, 44, 54, 55, 56, 57, 63, 65, 67, 68, 71, 77, 79, 84, 91, 95, 96, 105, 148, 234
	p-FBF	7	43, 44, 55, 70, 71, 91, 105

Spectra of FBF isomers collected in Month 2 were also compared to the FIBF reference spectra collected in Month 1. Once again, the numbers of ions responsible for discrimination were higher than the corresponding intra-month comparisons, ranging from 8 to 47 ions (Table 3.8). The identities of some of these ions were consistent with those observed during the intra-month comparisons such as *m/z* 43, 71, 90, 102, 118, 148, 164, 176, and 234. However, there were many ions which had not been observed as discriminating in the intra-month comparisons. Some of these ions were also observed for the FIBF inter-month comparisons in Table 3.7 such as *m/z* 55, 67, and 77, but there were higher mass ions such as *m/z* 141, 150, and 155 as well. The relative abundances of these new ions followed similar trends as shown in the FIBF to FIBF comparisons. Comparisons of FIBF and FBF comparison spectra collected in Month 2 to FBF reference spectra collected in Month 1 were also made and results are shown in Appendix Tables A3.8 and A3.9.

Table 3.8 Inter-month comparison of Month 1 FIBF as reference spectra and Month 2 FBF as comparison spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	m/z Values of Discriminating Ions
o-FIBF	o-FBF	13	43, 44, 67, 70, 96, 112, 113, 150, 154
	m-FBF	12	44, 45, 58, 71, 91, 95, 98, 102, 113, 118, 148, 190
	p-FBF	10	44, 71, 91, 95, 98, 102, 113, 118, 176, 234
m-FIBF	o-FBF	31	43, 44, 67, 70, 77, 90, 96, 102, 109, 110, 111, 112, 113, 116, 118, 122, 123, 124, 128, 130, 136, 137, 143, 144, 149, 150, 159, 160, 164, 165, 176
	m-FBF	11	43, 44, 45, 70, 91, 96, 111, 112, 113, 150, 164
	p-FBF	13	43, 44, 70, 96, 109, 110, 111, 113, 164, 165, 176, 234, 235
p-FIBF	o-FBF	47	43, 44, 54, 55, 56, 67, 70, 71, 77, 84, 90, 94, 96, 102, 105, 110, 111, 112, 113, 114, 116, 117, 118, 122, 124, 125, 128, 129, 130, 131, 132, 136, 138, 141, 142, 143, 144, 150, 152, 153, 155, 157, 159, 160, 164, 165, 166
	m-FBF	22	44, 45, 55, 56, 57, 58, 65, 67, 68, 71, 77, 84, 91, 94, 95, 96, 111, 112, 124, 136, 150, 164
	p-FBF	8	43, 44, 67, 91, 111, 112, 164, 176

The results from both FIBF and FBF comparisons, which demonstrate larger differences in spectral intensities over time due to instrument variation and tune conditions, provide further evidence of the need to analyze compounds under equivalent conditions. The instrument was used heavily across the three months during which data were collected for this study. As such, regular instrument maintenance was performed, including venting the mass spectrometer. An autotune was performed each day and the changes in the tune before and after the venting of the system affected the abundances of the ions in each spectrum. These differences in abundance can be seen visually in the spectra of *m*-FIBF from Month 1 and Month 2 in Figure 3.4, with ions having higher relative abundance in the Month 2 collection. Therefore, even though the electron multiplier response was similar for both months and the regression line data for predicting the standard deviation was similar, accurate comparisons were not possible.

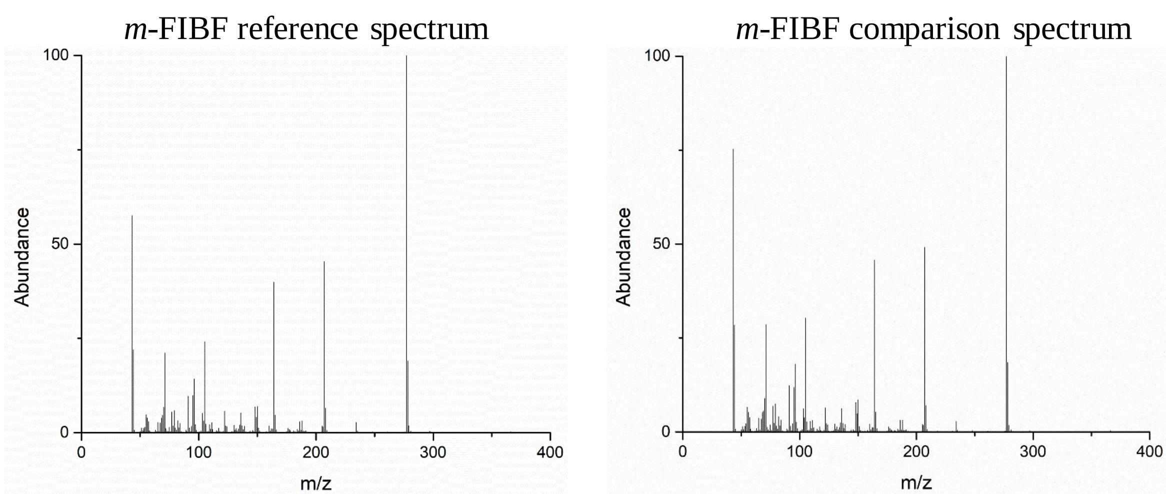

Figure 3.4 Comparison of the relative abundances of *m*-FIBF reference spectrum collected in Month 1 (left) and *m*-FIBF comparison spectrum collected in Month 2 (right)

3.5 Summary

The mass spectra of the positional isomers of FIBF and FBF were highly similar and distinction of the isomers was not possible based on visual assessment alone. However, statistical comparison of the spectra resulted in association and discrimination at the 99.9% confidence level according to the specific isomer.

Intra-month comparisons of the FIBF isomers across a three-month period allowed for the determination of common ions responsible for discrimination between each of the isomers. Comparisons in Months 1 – 3 were also made between FBF comparison spectra and FIBF reference spectra. This allowed for the determination of common discriminating ions between the two sets of isomers, as well as a demonstration of the the ability of the method to differentiate between the sets. The comparisons also showed evidence of the *ortho*-effect, with a greater number of discriminating ions present when either *o*-FIBF or *o*-FBF was compared to the other isomers.

Finally, results from the inter-month comparison between the Month 2 FIBF and FBF comparison spectra and the Month 1 FIBF reference spectra were discussed. In this case, association was not observed and the number of discriminating ions between the six isomers was higher than previously observed in the intra-month comparisons. These results highlighted the importance of analyzing the compounds to be used as the comparison spectra and the reference spectra under equivalent conditions for the most accurate association and discrimination. Even though the electron multiplier response was not statistically different in Months 1 and 2, it was not possible to produce accurate intra-month comparison results. This could be potentially due to an inaccurate method for standard deviation prediction or, more likely, that the mass spectrometer autotune resulted in different performance at low *m/z* values. The lowest *m/z* value produced by using perfluorobutylamine (PFTBA) along with the autotune method was *m/z* 69. Some of the common discriminating ions between the fentanyl isomers fell below this range: *m/z* 43 and *m/z* 44. Using a different tune compound or tune method may be more appropriate for the comparisons performed here.

Overall, the statistical comparison method proved successful in correctly associating and discriminating between FIBF and FBF isomers under equivalent conditions across the three-month study. Common discriminating ions were evaluated and future studies into their chemical structures could lead to information about why those ions were responsible for discrimination. In addition, a closer inspection and refinement of the procedure used to predict the standard deviations and perform the *t*-tests could lead to a more robust statistical comparison method.

APPENDIX

Table A3.1 PPMC coefficients of the pairwise comparisons of structural isomers of FIBF and FBF

	***o*-FIBF**	***m*-FIBF**	***p*-FIBF**
o-FBF	0.9841 ± 0.0017	0.9466 ± 0.0044	0.9686 ± 0.0032
m-FBF	0.9858 ± 0.0014	0.9790 ± 0.0033	0.9882 ± 0.0022
p-FBF	0.9880 ± 0.0012	0.9714 ± 0.0035	0.9848 ± 0.0023

Table A3.2 Comparison of two FIBF comparison spectrum data collections in Month 1 (A and B) to Month 1 FBF reference mass spectra at the 99.9% confidence level

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		A	B	A	B
***o*-FBF**	*o*-FIBF	1	2	43	43, 71
	m-FIBF	9	19	43, 71, 90, 102, 118, 144, 164, 165, 171	43, 71, 90, 95, 102, 110, 111, 112, 113, 118, 124, 130, 143, 144, 148, 157, 164, 165, 257
	p-FIBF	15	17	43, 71, 90, 95, 102, 112, 113, 118, 124, 141, 143, 144, 164, 165, 166	43, 71, 72, 90, 95, 102, 112, 113, 118, 124, 130, 138, 143, 144, 148, 164, 165
***m*-FBF**	*o*-FIBF	4	6	43, 94, 149, 164	43, 44, 122, 149, 164, 190
	m-FIBF	3	4	43, 71, 164	43, 44, 113, 164
	p-FIBF	4	4	43, 44, 112, 141	43, 44, 112, 122
***p*-FBF**	*o*-FIBF	5	5	43, 111, 130, 176, 234	43, 44, 122, 176, 234
	m-FIBF	6	8	43, 71, 164, 165, 176, 234	43, 71, 110, 164, 165, 176, 234, 235
	p-FIBF	4	5	43, 141, 164, 176	43, 44, 71, 164, 176

Table A3.3 Comparison of Month 1 FBF comparison spectra to corresponding FBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FBF**	*o*-FBF	0	–
	m-FBF	17	43, 44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 164, 165, 185
	p-FBF	14	43, 44, 71, 90, 95, 102, 118, 130, 143, 144, 164, 165, 234, 257
***m*-FBF**	*o*-FBF	16	44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 159, 164, 165
	m-FBF	0	–
	p-FBF	1	234
***p*-FBF**	*o*-FBF	11	71, 90, 102, 118, 130, 143, 144, 159, 164, 165, 234
	m-FBF	3	164, 176, 234
	p-FBF	0	–

Table A3.4 Comparison of Month 2 FIBF comparison spectra to corresponding Month 2 FBF reference spectra at the 99.9% confidence level

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**	***m/z* Values of Discriminating Ions**
***o*-FBF**	*o*-FIBF	2	43, 71
	m-FIBF	12	43, 71, 90, 91, 102, 105, 110, 113, 118, 144, 164, 165
	p-FIBF	16	43, 71, 72, 90, 95, 102, 112, 113, 118, 143, 144, 148, 159, 164, 165, 276
***m*-FBF**	*o*-FIBF	9	43, 44, 90, 122, 130, 143, 144, 149, 164
	m-FIBF	2	43, 71
	p-FIBF	2	43, 44
***p*-FBF**	*o*-FIBF	7	43, 44, 90, 130, 143, 176, 234
	m-FIBF	6	43, 71, 84, 164, 176, 234
	p-FIBF	5	43, 44, 71, 164, 176

Table A3.5 Comparison of Month 2 FBF comparison spectra to corresponding Month 2 FBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FBF**	*o*-FBF	0	–
	m-FBF	13	43, 44, 71, 90, 91, 95, 102, 118, 144, 148, 164, 165, 276
	p-FBF	10	43, 44, 71, 90, 95, 102, 118, 144, 164, 234
***m*-FBF**	*o*-FBF	13	44, 71, 90, 102, 110, 118, 122, 130, 143, 144, 149, 164, 165
	m-FBF	0	–
	p-FBF	1	234
***p*-FBF**	*o*-FBF	11	43, 44, 90, 130, 143, 144, 164, 176, 234
	m-FBF	1	234
	p-FBF	0	–

Table A3.6 Comparison of Month 3 FIBF comparison spectra to corresponding Month 3 FBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FBF**	*o*-FIBF	2	43, 164
	m-FIBF	8	43, 90, 102, 110, 144, 164, 165, 171
	p-FIBF	15	43, 71, 90, 91, 95, 102, 112, 113, 118, 124, 130, 143, 144, 164, 165
***m*-FBF**	*o*-FIBF	10	43, 44, 71, 90, 95, 102, 118, 122, 149, 164
	m-FIBF	2	43, 164
	p-FIBF	3	43, 44, 149
***p*-FBF**	*o*-FIBF	8	43, 44, 90, 102, 118, 130, 176, 234
	m-FIBF	5	43, 71, 164, 176, 234
	p-FIBF	4	43, 44, 71, 164

Table A3.7 Comparison of Month 3 FBF comparison spectra to corresponding Month 3 FBF reference spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	*m/z* Values of Discriminating Ions
***o*-FBF**	*o*-FBF	0	–
	m-FBF	12	43, 44, 71, 90, 102, 118, 122, 144, 148, 165, 171
	p-FBF	3	118, 164, 171
***m*-FBF**	*o*-FBF	11	90, 102, 110, 118, 122, 130, 144, 149, 164, 165, 171
	m-FBF	1	93
	p-FBF	0	–
***p*-FBF**	*o*-FBF	7	90, 102, 118, 130, 144, 164, 171
	m-FBF	3	43, 93, 234
	p-FBF	0	–

Table A3.8 Inter-month comparison of Month 1 FBF as reference spectra and Month 2 FIBF as comparison spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	m/z Values of Discriminating Ions
***o*-FBF**	*o*-FIBF	34	43, 44, 50, 51, 53, 54, 55, 56, 57, 63, 65, 67, 68, 70, 71, 72, 75, 77, 78, 79, 82, 83, 84, 91, 94, 95, 96, 103, 104, 105, 106, 111, 122, 205
	m-FIBF	24	43, 44, 68, 70, 71, 75, 77, 79, 82, 95, 96, 102, 103, 104, 105, 113, 118, 135, 144, 162, 164, 165, 205, 257
	p-FIBF	37	43, 44, 50, 51, 52, 53, 54, 55, 56, 57, 65, 68, 70, 71, 75, 77, 78, 79, 91, 92, 95, 96, 97, 102, 103, 105, 112, 113, 118, 135, 143, 144, 148, 159, 164, 165, 181
***m*-FBF**	*o*-FIBF	40	43, 51, 52, 54, 55, 56, 57, 65, 67, 68, 70, 76, 77, 78, 79, 82, 83, 91, 94, 96, 103, 104, 105, 106, 109, 110, 111, 116, 117, 122, 128, 130, 132, 144, 149, 164, 165, 185, 205, 208
	m-FIBF	25	43, 52, 54, 55, 56, 57, 63, 65, 68, 70, 71, 72, 77, 78, 79, 82, 84, 91, 95, 96, 98, 103, 105, 106, 122
	p-FIBF	16	43, 55, 57, 68, 70, 71, 77, 79, 91, 96, 105, 109, 111, 121, 122, 149
***p*-FBF**	*o*-FIBF	40	43, 51, 53, 54, 55, 56, 57, 65, 67, 68, 70, 76, 77, 78, 79, 84, 91, 94, 96, 103, 104, 105, 106, 111, 116, 117, 122, 130, 143, 144, 149, 157, 159, 164, 176, 181, 185, 205, 208, 234
	m-FIBF	27	43, 44, 51, 53, 54, 55, 56, 57, 58, 63, 65, 68, 71, 72, 77, 79, 82, 84, 91, 95, 96, 105, 148, 164, 176, 205, 234
	p-FIBF	16	43, 51, 55, 57, 68, 70, 71, 72, 77, 79, 91, 96, 105, 111, 122, 181

Table A3.9 Inter-month comparison of Month 1 FBF as reference spectra and Month 2 FBF as comparison spectra at the 99.9% confidence level

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions	m/z Values of Discriminating Ions
***o*-FBF**	*o*-FBF	42	43, 44, 45, 50, 51, 52, 53, 54, 55, 56, 57, 65, 67, 68, 70, 71, 75, 77, 78, 79, 80, 82, 83, 84, 91, 92, 94, 95, 96, 97, 98, 103, 104, 105, 106, 109, 111, 122, 136, 149, 150, 164
	m-FBF	34	43, 44, 51, 53, 54, 55, 56, 57, 63, 65, 67, 68, 70, 71, 75, 77, 78, 79, 82, 84, 91, 92, 95, 96, 102, 103, 105, 111, 118, 135, 144, 148, 164, 214
	p-FBF	21	43, 44, 54, 55, 56, 57, 65, 70, 71, 79, 91, 96, 102, 103, 105, 111, 118, 135, 148, 234, 257
***m*-FBF**	*o*-FBF	53	43, 51, 53, 54, 55, 56, 57, 65, 67, 68, 70, 76, 77, 79, 82, 83, 89, 90, 91, 94, 96, 97, 102, 103, 104, 105, 106, 109, 110, 111, 112, 116, 117, 118, 122, 123, 124, 128, 130, 131, 132, 136, 137, 142, 143, 144, 149, 150, 156, 159, 160, 164, 165
	m-FBF	25	43, 44, 51, 52, 54, 55, 56, 57, 65, 67, 68, 70, 71, 77, 79, 82, 84, 91, 94, 95, 96, 98, 103, 105, 111
	p-FBF	17	43, 44, 51, 55, 70, 91, 96, 98, 105, 109, 110, 111, 122, 149, 164, 176, 234
***p*-FBF**	*o*-FBF	45	43, 51, 53, 54, 55, 56, 57, 64, 67, 68, 70, 77, 79, 80, 82, 84, 90, 94, 96, 102, 105, 110, 111, 112, 116, 117, 118, 122, 123, 124, 128, 129, 130, 131, 132, 136, 143, 144, 149, 159, 160, 164, 165, 214, 234
	m-FBF	26	43, 44, 51, 53, 54, 55, 56, 57, 58, 65, 68, 71, 77, 79, 82, 84, 91, 94, 95, 96, 98, 103, 105, 111, 148, 234

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IV. Further Investigation of a Refined Approach to Predict Standard Deviation

While the overall results of the intra-month comparisons of the fluoroisobutyryl fentanyl (FIBF) and fluorobutyryl fentanyl (FBF) isomers in Months 1-3 led to successful association and discrimination in most cases, there were some comparisons that resulted in incorrect associations (false positives) and incorrect discriminations (false negatives). Additionally, the inter-month comparisons between Month 1 and Month 2 resulted in no association and discriminations with many ions responsible. Following the results demonstrated in Chapter 3, further investigation into the regression plots was made in order to refine the method of standard deviation prediction to more accurately model the response of the electron multiplier and increase confidence in the statistical comparisons of the FIBF and FBF isomers.

4.1 Investigation of Regression Lines

In order to perform the statistical comparisons of FIBF and FBF isomers, a mathematical model was previously created to predict the standard deviations used in the *t*-test calculation. This method of standard deviation prediction is based upon the counting statistics of the electron multiplier response (Sections 1.3 and 2.3). Using the spectral data from the set of normal (*n*-) alkanes analyzed in replicate and at different concentrations, regression plots were generated for each month of analysis as shown in Figure 4.1 A-C. The slopes ranged from 0.6468 to 0.7190 and the coefficient of linear fit (R^2) ranged from 0.86 to 0.91.

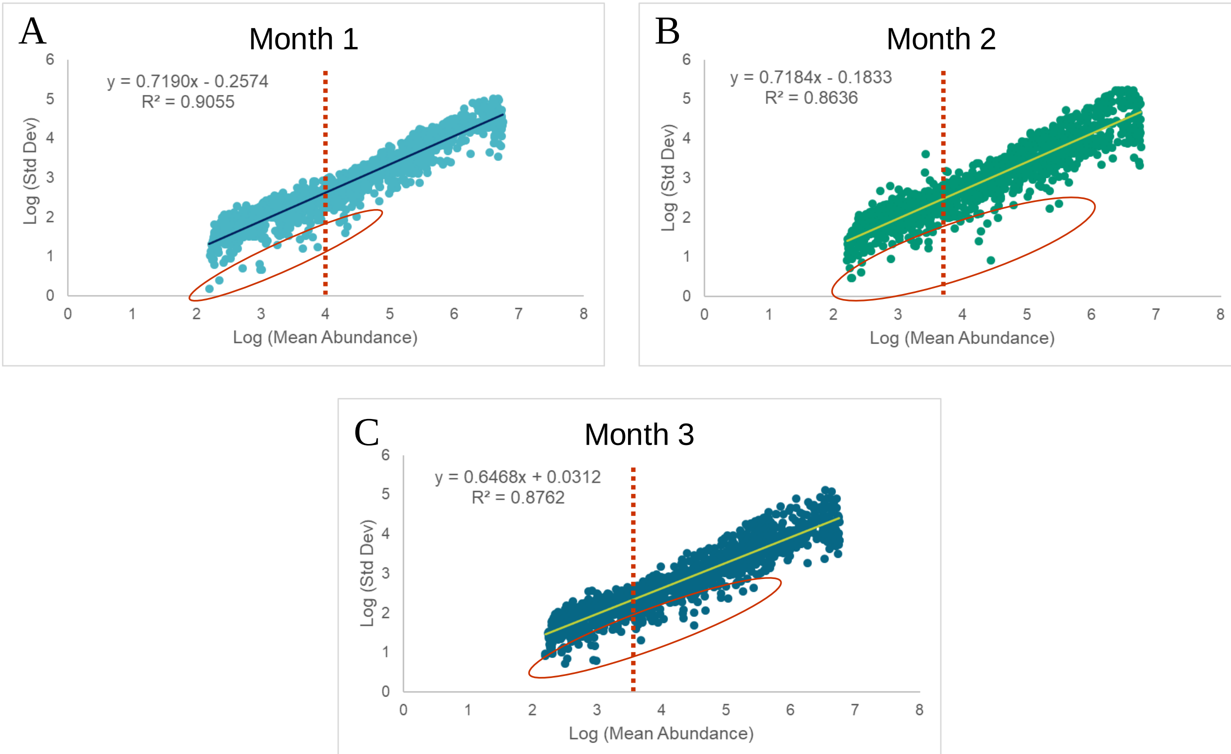

Figure 4.1 Regression line plot results from Month 1 (A), Month 2 (B), and Month 3 (C). Red ellipses highlight data points that were tested for outliers using the Z-score test and the red dotted lines indicate the division points for multiple slope comparisons.

4.1.1 Testing for Outliers

Upon closer inspection of the regression plots in Figure 4.1, there were a number of potential outliers in each plot, especially towards the lower abundance end of each regression line (highlighted in the red ellipses in Figure 4.1). Using Z-scores to statistically assess for outliers, scores were assigned to each data point in the regression plots. Data points with a calculated Z-score of greater than ±2 were removed from the regression line and the slope and y-intercept of each line were recalculated.[1] While the standard cut-off value for finding outliers are Z-scores of ±3, in these data sets there were no data points that fell within that category.[1] Therefore, any data points that were two standard deviations above or below the mean were removed for this test. For the Month 1 data, there were a total of 1270 data points and of these, 27 data points were defined as outliers following the Z-score test. For Month 2, there were a total of 1347 data points of which 46 were defined as outliers. For the Month 3 data, there were 1270 total data points of which 34 were outliers. A comparison of the slope and y-intercept from the original regression lines and the lines with outliers removed is shown in Table 4.1.

Table 4.1 Regression line slope and y-intercept results for Months 1-3 before and after the removal of calculated outliers

Regression Month	**Slope**		**y-Intercept**	
	Original	Outliers Removed	Original	Outliers Removed
Month 1	0.7190	0.7020	-0.2574	-0.1812
Month 2	0.7184	0.6881	-0.1833	-0.0676
Month 3	0.6468	0.6251	+0.0312	+0.1208

Focusing primarily on the slope differences after the removal of the outliers, the slopes in each month decreased. However, for the shot-noise limited range, the expected slope is close to 0.5, and even after the removal of the outliers defined by the Z-score test, none of the slopes for Months 1-3 decreased to close to 0.5.

Statistical comparisons of the FIBF and FBF spectra data were performed again, now using the refined regression coefficients obtained after removing outliers to predict standard deviations. Results from the comparisons using the refined regression coefficients were similar to the results from comparisons prior to the removal of the outliers. For example, in the Month 1 comparisons between FIBF reference spectra and FIBF comparison spectra, association of the corresponding spectra of *o*- and *m*-FIBF was still possible at the 99.9% confidence level using the refined coefficients. In terms of discrimination, most of the comparisons among the FIBF isomers resulted in the same number and identities of discriminating ions. There were two instances in which the number of discriminating ions varied. The comparison between the *p*-FIBF comparison spectrum and the *o*-FIBF reference spectra resulted in 10 discriminating ions when using the original regression coefficients and only 9 ions when using the refined coefficients after outlier removal. The one ion that was different was *m/z* 143, which had a t_{calc} value of 13.169 in the original comparison and a t_{calc} value of 12.589 in the refined comparison which were compared to a t_{crit} value of 12.924 in both situations.

The *o*-FIBF comparison spectrum to the *m*-FIBF reference spectra also resulted in a different number of discriminating ions with the removal of outliers from the regression line. In the original comparison, there were only 8 discriminating ions, but in the comparison using the refined regression coefficients, there were 9 discriminating ions. The ion that was present in the refined comparison but not the original one was *m/z* 102, which had t_{calc} values of 21.805 and 21.187 in the original and refined comparisons, respectively. However, in this case, the t_{crit} values changed between the two comparisons – from 31.599 in the original comparison to 12.924 in the comparison after removing outliers. Therefore, the differences in regression line coefficients pre- and post-outlier removal did affect the predicted standard deviations of the reference spectra and comparison spectra slightly as well as changing the degrees of freedom results, which in-turn affected the t_{calc} and t_{crit} values of each *m/z* value in the spectra being statistically compared. However, these changes did not affect the overall results. No comparison between FIBF or FBF isomers resulted in a different overall result of association or discrimination due to the removal of the outliers. In addition, the differences in the numbers of discriminating ions did not vary greatly between the two types of comparisons, as shown in the

Month 1 comparison example. Results from all other comparisons can be found in Appendix Tables A4.1 – A4.12.

Because of the similar results, all comparisons moving forward were performed using the original regression coefficients, without the extra step of removing outliers. This is also a simpler and more streamlined approach that is more practical for applying the method to casework in a forensic laboratory.

4.1.2 Investigating Different Linear Regions Within Each Plot

Upon further inspection of the regression lines for each month, it appeared that within each plot there may be two linear regions with different slopes, which are indicated on either side of the red dotted lines in Figure 4.1. The first region includes a lower abundance region in which the standard deviation is proportional to abundance in a manner similar to that expected for shot-noise limits with a slope closer to 0.5. The second region includes a higher abundance portion expected for signal-to-noise scaling directly with signal with a slope closer to 1.0 (proportional noise region). Using two slopes, or a segmented regression line, to more accurately predict the standard deviation as a function of abundance could result in more accurate *t*-values, and therefore, more accurate comparisons. Based on visual inspection of each regression line, the abundance value to use as the breakpoint was manually selected, which is denoted by the red dotted lines in Figures 4.1 and 4.2.

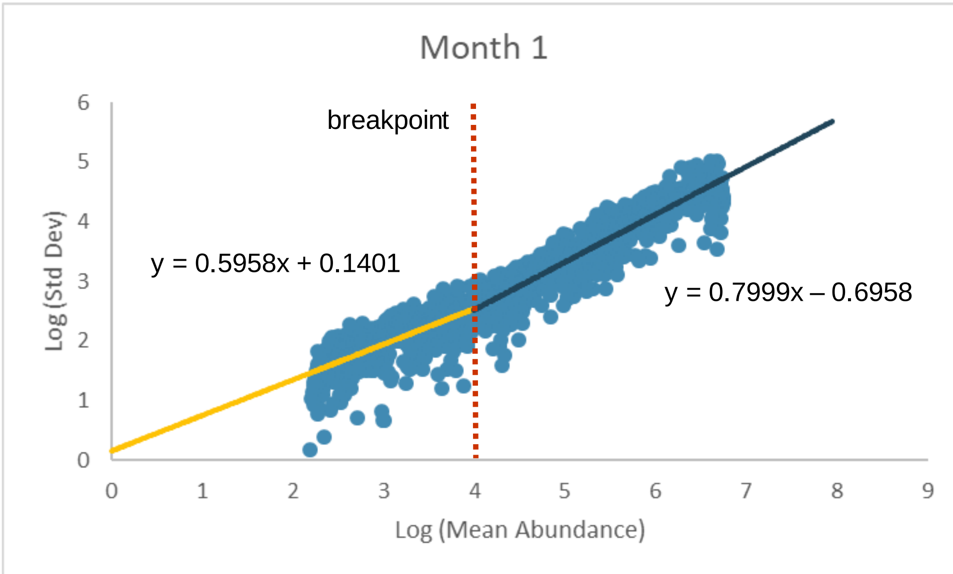

Figure 4.2 Plot of the two slope regions in the Month 1 regression, with a lower abundance region on the left side of the red line and a higher abundance region on the right.

The breakpoint was selected to ensure that the lower abundance line had a slope as close to 0.5 as possible. Then regression analysis was performed separately on the lower and upper range, resulting in two regression equations representing the two different linear regions of the graph. The slope and y-intercept coefficients from each regression were determined (Table 4.2). The x-value at which each line intersected (intersection point) was calculated and used to determine the abundance at which the regression lines were divided into two regions (Table 4.2).[2] As demonstrated in Table 4.2, the slopes for the lower abundance regions of each line decreased to closer to 0.5, with the slopes for the higher abundance regions approaching 1.0.

Table 4.2 Regression line slope and y-intercept results for Months 1-3 before and after division including the abundance and x-value point at which the division into two regions was made.

Regression Month	Slope		y-Intercept		Intersection Point	Abundance at Break
	1-Slope	2-Slope	1-Slope	2-Slope		
Month 1	0.7190	0.5958 0.7999	-0.2574	+0.1401 -0.6958	4.096	12460.7
Month 2	0.7184	0.6445 0.7544	-0.1833	+0.0489 -0.3746	3.854	7136.79
Month 3	0.6468	0.5574 0.6760	+0.0312	+0.3089 -0.1245	3.654	4511.28

The automated spreadsheet template used to perform the statistical comparisons was modified to include two regression slopes, two intercepts, and the user-defined cut-off abundance point in order to statistically compare spectra using the refined model of electron multiplier response. For abundances less than the threshold value that was defined, the predicted standard deviations for each *m/z* value were calculated using the regression coefficients corresponding to the low abundance region of the regression data. For abundances greater than the breakpoint, the standard deviations were predicted using the coefficients corresponding to the high abundance region of the regression data. For example, for Month 1 data (as shown in Figure 4.2), the standard deviation associated with abundances less than $10^{4.096}$ (12460.7) was calculated using a slope of 0.5958 and an intercept of 0.1401, whereas for abundance values greater than $10^{4.096}$, standard deviations were calculated using a slope of 0.7999 and an intercept of -0.6958.

The predicted standard deviations at the threshold abundance of 150 counts were recorded for each month using both the two-slope calculation method and the one-slope calculation. This was done in order to demonstrate the effect of predicting standard deviations using the two types of calculations on the statistical comparison results. The results are shown in Table 4.3. Using the coefficients from two regression lines, the predicted standard deviations increased for each month. It should be noted that this increase in predicted standard deviation is larger than the difference observed from removing outliers in the previous section. Those results can also be found in Table 4.3. Because standard deviation is included in the *t*-test calculations, it was necessary to investigate whether the refined standard deviations calculated using a two-slope regression affect the overall association and discrimination of the FIBF and FBF spectra.

Table 4.3 Differences in the predicted standard deviations of the threshold abundances using the original prediction method, the original method without outliers, and the refined prediction method

Regression Month	Standard Deviation Original	Standard Deviation Outlier Removal	Standard Deviation Refined
Month 1	20.2866	22.2032	27.3285
Month 2	23.9885	26.9010	28.2742
Month 3	27.4597	30.2743	33.2547

*standard deviations were calculated based on an abundance value of 150

4.2 Intra-Month Comparisons of FIBF and FBF Spectra to FIBF Reference Spectra Using the Refined Method of Standard Deviation Prediction

Comparisons of the same data collections of FIBF and FBF spectra as used in Chapter 3 were performed using the refined method of standard deviation prediction. The effect of using two slopes to better fit the regions of lower and higher abundance values on the predicted standard deviation results and the t_{calc} and t_{crit} values were investigated. This was tested by comparing the results from the comparisons using the refined method to the original results to demonstrate any differences in the association and discrimination observed. Similar to Chapter 3, only FIBF reference spectra comparisons are shown in this chapter, but FBF reference spectra comparisons are included in the Appendix Tables A4.13 – A4.19.

4.2.1 Month 1 FIBF and FBF Spectra Compared to Month 1 FIBF Reference Spectra Using the Refined Method to Predict Standard Deviation

The FIBF comparison spectra collected in Month 1 were compared to the corresponding Month 1 FIBF reference spectra using the refined method to predict standard deviation. As mentioned in Chapter 3, two sets of Month 1 comparison spectra were collected (Month 1A and Month 1B) to demonstrate the ability of the method to statistically compare spectra analyzed on different days to the same reference spectra under equivalent conditions and still yield similar results. Table 4.4 shows the results for the comparison between Month 1A comparison spectra to corresponding FIBF reference spectra for both the original comparisons and the refined method comparisons. While the number of discriminating ions was different between the two comparisons, the overall results, whether association or discrimination was made, were unchanged. Corresponding spectra of *o*-FIBF, *m*-FIBF, and *p*-FIBF were statistically associated at the 99.9% confidence level when using the refined method with the same maximum and minimum random-match probabilities (P_{max} and P_{min}) for each comparison as observed during the original comparisons.

In terms of discrimination, the Month 1A comparison FIBF spectra were still discriminated from the FIBF reference spectra in all cases (Table 4.4) at the 99.9% confidence level. However, the refined method resulted in a higher number of discriminating ions in all cases except for the comparison between *p*-FIBF reference spectra and the *o*-FIBF comparison spectrum, where the number of ions remained the same (3). In most of these instances, the *m/z* values of the discriminating ions remained constant between the original comparisons and the comparisons using the refined method, with the refined method only identifying additional discriminating ions.

Table 4.4 Comparisons of Month 1A FIBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	9	14	43, 71, 90, 102, 118, 144, 149, 164, 165	43, 44, 71, 90, 95, 102, 118, 122, 144, 148, 149, 164, 165, 171
	p-FIBF	9	10	71, 90, 102, 112, 118, 130, 144, 164, 165	71, 84, 90, 102, 112, 118, 130, 144, 164, 165
***m*-FIBF**	*o*-FIBF	6	13	71, 90, 111, 148, 164, 165	71, 90, 95, 110, 111, 118, 122, 130, 144, 148, 149, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	1	2	234	84, 234
***p*-FIBF**	*o*-FIBF	3	3	71, 164, 234	71, 164, 234
	m-FIBF	2	3	164, 234	44, 164, 234
	p-FIBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

The refined method comparisons involving *o*-FIBF as either the comparison spectrum or reference spectra resulted in new common discriminating ions including *m/z* 95 and *m/z* 122. In the case of *m/z* 95, this ion was present as a new discriminating ion for the comparisons of *o*-FIBF to *m*-FIBF. In both situations, the predicted standard deviations of the reference and comparison spectra for that ion decreased when using the refined method. For example, in the comparison of the *m*-FIBF comparison spectrum to the *o*-FIBF reference spectra, the predicted standard deviation of *m/z* 95 in the *m*-FIBF comparison spectrum was 3.03×10^{-3} in the original comparison and 2.50×10^{-3} in the refined comparison. The effect of the decrease in predicted standard deviations of that ion resulted in an increase in the t_{calc} value without also increasing the t_{crit} value. In the original comparison, the t_{calc} value was 12.645 compared to a t_{crit} value of 12.924. Using the refined method, the t_{calc} value increased to 15.044, which was greater than the t_{crit} value and the null hypothesis was no longer accepted at that *m/z* value. This resulted in an additional discriminating ion. This was also the case for the ion at *m/z* 122, where the predicted standard deviations in both the comparison and reference spectra decreased, which increased the t_{calc} value to greater than the t_{crit} value. The comparisons using the refined method that involved *m*-FIBF contained a new discriminating ion at *m/z* 44, where the t_{calc} value increased from 12.368 to 13.510. The new discriminating ion observed when comparing *p*-FIBF using the refined method was *m/z* 84, where the t_{calc} value increased from 10.938 to 13.470.

Comparisons of Month 1B FIBF comparison spectra to corresponding Month 1 reference spectra were also performed using the refined method and results were related to the one-slope comparisons in Table 4.5. As with the Month 1A comparisons above, similar overall results of association and discrimination were observed between the two different types of comparisons. Corresponding spectra of *o*-FIBF and *m*-FIBF were associated at the 99.9% confidence level

(Table 4.5). The same P_{max} and P_{min} were observed for each association as for the original comparisons as well. For corresponding spectra of *p*-FIBF, association was not attained using either method. In both instances, one ion (*m/z* 366) was responsible for the incorrect discrimination or false negative.

In terms of discrimination, the Month 1B comparison FIBF spectra were still discriminated from the FIBF reference spectra in all cases at the 99.9% confidence level (Table 4.4). Using the refined method to predict standard deviation resulted in a higher number of discriminating ions in all cases except for the comparison between *m*-FIBF reference spectra and the *p*-FIBF comparison spectrum where the number of ions remained the same (2). For the Month 1B comparisons, the *m/z* values of the discriminating ions remained constant between the two types of comparisons in all cases, with the refined method results only adding to the existing list.

Trends in the Month 1B data were not as obvious as those observed in the Month 1A comparisons. The refined method comparisons involving *o*-FIBF as either the reference spectra or the comparison spectrum did not result in any common ions that were present in all comparisons involving that isomer. However, some of the newly added discriminating ions resulting from the refined comparison of *o*-FIBF to the other isomers included *m/z* 102, 112, and 130. The refined method comparisons that involved *m*-FIBF resulted in new discriminating ions including *m/z* 122 and *m/z* 176, but again, no ions were commonly observed in all of the comparisons that involved that isomer. And finally, the refined method comparisons involving *p*-FIBF did contain a common discriminating ion, *m/z* 234, in all comparisons that included that isomer.

Table 4.5 Comparisons of Month 1B FIBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	13	20	70, 71, 90, 102, 110, 111, 122, 130, 144, 149, 164, 165, 185	70, 71, 90, 102, 110, 111, 117, 118, 122, 123, 130, 132, 143, 144, 149, 160, 164, 165, 185
	p-FIBF	10	14	71, 84, 90, 111, 112, 130, 143, 144, 164, 165	71, 84, 90, 94, 111, 112, 124, 130, 143, 144, 164, 165, 184, 234
***m*-FIBF**	*o*-FIBF	8	12	43, 71, 90, 95, 118, 148, 164, 165	43, 71, 90, 95, 102, 118, 122, 148, 149, 164, 165, 181
	m-FIBF	0	0	–	–
	p-FIBF	2	2	84, 234	84, 234
***p*-FIBF**	*o*-FIBF	2	6	71, 164	71, 95, 112, 130, 164, 234
	m-FIBF	4	7	70, 110, 164, 234	70, 110, 111, 164, 165, 176, 234
	p-FIBF	1	1	366	366

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Additionally, the FBF comparison spectra collected in Month 1 were compared to the Month 1 FIBF reference spectra using the two-slope comparison method to demonstrate the ability of the method to discriminate between the two sets of isomers. Results from both the original comparisons and those performed using the refined method of standard deviation prediction are shown in Table 4.6. All discriminations were made at the 99.9% confidence level and the overall results between the two comparison methods were similar. The refined method comparisons resulted in a higher number of discriminating ions in all comparisons except the comparison between the *m*-FIBF reference spectra and the *m*-FBF comparison spectrum, which remained the same (3).

For the comparisons to the *o*-FIBF reference spectra, some common additional ions observed in the refined comparisons to the FBF isomers included *m/z* 160 and *m/z* 205. For both ions, the predicted standard deviation in both the comparison spectra and reference spectra decreased. This lead to an increase in the t_{calc} value of *m/z* 160 from 11.133 to 13.796 and an increase in the t_{calc} value of *m/z* 205 from 10.548 to 13.332. In both cases, the increase in t_{calc} values lead to rejection of the null hypothesis in the refined method comparisons. In the comparison between the *o*-FIBF reference spectra and the *o*-FBF comparison spectrum, one ion (*m/z* 164) which had been responsible for discrimination in the original comparisons was not observed as a discriminating ion using the refined method. In this instance, the standard deviations increased from the original comparison to the refined method, resulting in a t_{calc} value of 12.169 using the refined method instead of 13.231. The new t_{calc} value was less than the t_{crit} value of 12.924 and the null hypothesis was accepted, meaning the abundances of that ion in the spectra being compared were statistically indistinguishable using the refined method of standard deviation prediction.

Table 4.6 Comparisons of Month 1B FBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FBF	3	4	43, 113, 164	43, 113, 176, 205
	m-FBF	12	22	43, 44, 90, 105, 111, 122, 130, 144, 149, 164, 190, 208	43, 44, 70, 77, 90, 95, 105, 110, 111, 113, 117, 122, 130, 144, 149, 160, 164, 165, 190, 204, 205, 208
	p-FBF	11	19	43, 44, 84, 90, 122, 130, 144, 176, 185, 208, 234	43, 44, 84, 90, 94, 102, 105, 111, 118, 122, 130, 144, 160, 176, 185, 205, 208, 234, 235
***m*-FIBF**	*o*-FBF	14	19	43, 71, 90, 102, 110, 112, 113, 118, 143, 144, 149, 164, 165, 176	43, 71, 90, 95, 102, 110, 112, 113, 118, 130, 136, 143, 144, 148, 149, 164, 165, 171, 176
	m-FBF	3	3	43, 71, 113	43, 71, 113
	p-FBF	7	8	43, 71, 84, 164, 176, 234, 235	43, 71, 84, 164, 165, 176, 234, 235

Table 4.6 (cont'd.)

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***p*-FIBF**	*o*-FBF	19	24	43, 71, 72, 90, 95, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 159, 164, 165, 166	43, 71, 72, 90, 95, 102, 110, 112, 113, 116, 118, 124, 128, 130, 131, 136, 138, 143, 144, 152, 159, 164, 165, 166
	m-FBF	6	11	43, 44, 71, 122, 149, 366	43, 44, 71, 109, 112, 113, 122, 149, 218, 234, 366
	p-FBF	3	4	43, 44, 71	43, 44, 71, 176

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

For comparisons to *m*-FIBF reference spectra, there were no real trends observed in the additional discriminating ions defined by using the refined method. The same was the case for comparisons to the *p*-FIBF reference spectra. However, for all nine comparisons between the FIBF reference spectra and FBF comparison spectra, ions such as *m/z* 95, 110, 176, and 205 were among some of the most commonly observed additions. In each of these cases, the predicted standard deviations of the abundances of those ions decreased when using the refined comparison method, leading to a large enough increase in the t_{calc} value which resulted in a rejection of the null hypothesis.

4.2.2 Month 2 FIBF and FBF Spectra Compared to Month 2 FIBF Reference Spectra Using the Refined Method to Predict Standard Deviation

Comparisons were performed using the refined method of standard deviation prediction on the Month 2 data involving FIBF reference spectra to corresponding FIBF comparison spectra. Results were compared to the corresponding original comparisons as shown in Table 4.7. Overall, the results between the two types of comparisons were similar, with association at the 99.9% confidence level for corresponding spectra of all *o*-, *m*-, and *p*-FIBF isomers with the same P_{max} and P_{min} values in each instance as those observed from the original comparisons. Each isomer of FIBF was also discriminated from the other two isomers at the 99.9% confidence level using both types of comparisons.

In the comparisons using the refined method, a higher number of discriminating ions was observed for the comparisons of the *o*-FIBF reference spectra to both *m*- and *p*-FIBF comparison spectra and the *p*-FIBF reference spectra to the *o*-FIBF comparison spectrum (Table 4.7). All other comparisons resulted in the same number and identity of observed discriminating ions using each type of comparison. All cases that involved an increase in the number of discriminating ions contained the same original discriminating ions.

Because many of the comparisons resulted in the same number of discriminating ions, fewer trends were observed. Comparisons involving *o*-FIBF resulted in a common additional ion at *m/z* 144. The predicted standard deviations of that ion decreased, leading to an increase in the t_{calc} value from 12.139 to 13.379, while the t_{crit} value remained the same at 12.924.

Table 4.7 Comparisons of Month 2 FIBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	8	10	43, 44, 71, 95, 102, 111, 148, 164	43, 44, 71, 95, 102, 111, 118, 144, 148, 164
	p-FIBF	5	6	71, 111, 112, 164, 234	71, 111, 112, 130, 164, 234
***m*-FIBF**	*o*-FIBF	7	7	71, 90, 102, 118, 149, 164, 165	71, 90, 102, 118, 149, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	1	1	234	234
***p*-FIBF**	*o*-FIBF	5	8	71, 90, 130, 143, 164	71, 90, 112, 130, 143, 144, 164, 165
	m-FIBF	5	5	43, 44, 71, 84, 234	43, 44, 71, 84, 234
	p-FIBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Comparisons of the Month 2 FIBF reference spectra to the corresponding FBF comparison spectra were also performed using the refined method of standard deviation prediction. Results from the original comparisons and the comparisons using the refined method are compared in Table 4.8. Discrimination was still possible at the 99.9% confidence level and most comparisons resulted in a higher number of discriminating ions when using the refined method. The two types of comparisons of *m*-FIBF reference spectra to both *m*- and *p*-FBF resulted in the same number and identity of discriminating ions, 3 and 5, respectively. All ions that were responsible for discrimination in the original comparisons were also defined as discriminating in the refined comparison results. Overall, no trends in the identities of the new discriminating ions were observed; however, some ions were commonly observed as additional discriminating ions in both Month 1 (Table 4.5) and Month 2 (Table 4.7). These ions included *m/z* 95, 102, 111, 112, 171, and 176. For *m/z* 95, 102, and 171, the t_{crit} value in the original comparisons was 31.599 and decreased to 12.924 using the refined standard deviation prediction method. While the t_{calc} values also changed, the reason for the difference in the hypothesis test result was the change in t_{crit} values. For *m/z* 111, 112, and 176, the predicted standard deviations all decreased, causing the t_{calc} values to all increase to be greater than 12.924, and resulting in the rejection of the null hypothesis at those *m/z* values.

Table 4.8 Comparisons of Month 2 FBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FBF	4	5	44, 111, 113, 164	43, 44, 111, 113, 164
	m-FBF	7	10	44, 71, 95, 111, 118, 122, 148	44, 71, 90, 95, 102, 111, 118, 122, 144, 148
	p-FBF	7	8	43, 44, 90, 102, 118, 176, 234	43, 44, 90, 102, 111, 118, 176, 234

Table 4.8 (cont'd.)

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***m*-FIBF**	*o*-FBF	13	17	43, 90, 102, 110, 112, 113, 118, 136, 143, 144, 149, 164, 165	43, 90, 95, 102, 110, 112, 113, 118, 124, 136, 143, 144, 149, 159, 164, 165, 171
	m-FBF	3	3	43, 44, 164	43, 44, 164
	p-FBF	5	5	43, 71, 164, 176, 234	43, 71, 164, 176, 234
***p*-FIBF**	*o*-FBF	17	18	43, 44, 71, 90, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 150, 164, 165	43, 44, 71, 90, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 150, 164, 165, 166
	m-FBF	2	5	43, 44	43, 44, 84, 112, 124
	p-FBF	3	4	43, 44, 164	43, 44, 164, 176

4.2.3 Month 2 FIBF and FBF Spectra Compared to Month 2 FIBF Reference Spectra Using the Refined Method to Predict Standard Deviation

Comparisons using the refined method of standard deviation prediction were also performed on FIBF comparison spectra collected in Month 3 to the corresponding Month 3 FIBF reference spectra. A comparison of the original results to those with the refined method is shown in Table 4.9. As shown with Month 1 and 2 data above, similar overall results of association and discrimination were observed between the two comparison methods. All associations were possible at the 99.9% confidence level for corresponding spectra of *o*-, *m*-, and *p*-FIBF for both methods. Additionally, the same P_{max} and P_{min} values were observed for each association as observed for the original comparisons.

In terms of discrimination, all isomers were discriminated at the 99.9% confidence level using the refined comparison method. In the original comparison, there was an incorrect association between the *m*-FIBF reference spectra and the *p*-FIBF comparison spectrum (false positive), which was reversed to a correct discrimination with one ion (*m/z* 234) responsible for discrimination. In the original comparison, the standard deviations of the abundances of *m/z* 234 in the comparison spectrum and reference spectra were 1.21×10^{-3} and 1.61×10^{-3}, respectively. Using the refined method, the standard deviations decreased to 1.14×10^{-3} and 1.47×10^{-3}, respectively. This resulted in an increase of the t_{calc} value from 12.364 to 13.377, meaning t_{calc} was greater than t_{crit} using the refined method. Therefore, the two isomers were no longer incorrectly associated to one another. All but one discrimination comparison resulted in a higher number of discriminating ions using the refined method. The exception was the comparison between *p*-FIBF reference spectra and the *m*-FIBF comparison spectrum, where the number of discriminating ions remained the same at 2. One observed trend in the Month 3 data was the addition of *m/z* 122 when comparing *o*- and *m*-FIBF to one another. Using the refined method of standard deviation prediction, the standard deviations of the abundances of *m/z* 122 in both the comparison spectrum and reference spectra decreased, causing an increase in the t_{calc} values.

Table 4.9 Comparisons of Month 3 FIBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	5	7	71, 90, 102, 164, 165	71, 90, 95, 102, 122, 164, 165
	p-FIBF	3	5	71, 95, 164	71, 95, 112, 130, 164
***m*-FIBF**	*o*-FIBF	7	8	71, 95, 102, 118, 149, 164, 165	71, 95, 102, 118, 122, 149, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	0	1	–	234
***p*-FIBF**	*o*-FIBF	5	6	71, 90, 95, 144, 164	71, 90, 95, 130, 144, 164
	m-FIBF	2	2	164, 234	164, 234
	p-FIBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

A final comparison between FBF comparison spectra collected in Month 3 and the corresponding FIBF reference spectra was made using the refined comparison method (Table 4.10). Consistent with results from Months 1 and 2, similar discrimination results were observed with the two types of comparison methods. All isomers were discriminated at the 99.9% confidence level. The refined comparison results contained either the same number of discriminating ions or a higher number of discriminating ions compared to the original comparisons. Although no trends were observed in the identities of the additional discriminating ions within the Month 3 data, only two ions *m/z* 102 and *m/z* 176 were observed in the FBF to FIBF comparisons across all three months. For both ions, the t_{calc} values increased to greater than the t_{crit} values of 12.924 for all months.

Table 4.10 Comparisons of Month 3 FBF comparison spectra and corresponding FIBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FIBF**	*o*-FBF	2	2	43, 164	43, 164
	m-FBF	8	9	43, 44, 71, 93, 95, 122, 148, 164	43, 44, 71, 90, 93, 95, 122, 148, 164
	p-FBF	3	5	43, 44, 234	43, 44, 102, 176, 234
***m*-FIBF**	*o*-FBF	6	9	43, 90, 102, 118, 164, 165	43, 90, 102, 110, 118, 130, 149, 164, 165
	m-FBF	4	4	43, 44, 93, 164	43, 44, 93, 164
	p-FBF	3	5	43, 164, 234	43, 44, 164, 176, 234
***p*-FIBF**	*o*-FBF	14	14	43, 71, 90, 95, 102, 112, 118, 124, 130, 136, 143, 144, 164, 165	43, 71, 90, 95, 102, 112, 118, 124, 130, 136, 143, 144, 164, 165
	m-FBF	2	3	43, 44	43, 44, 93
	p-FBF	4	4	43, 44, 111, 164	43, 44, 111, 164

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

4.4 Summary

Since the inter-month comparisons between Month 1 and Month 2 FIBF and FBF data resulted in no correct association and discrimination with an unlikely number of ions responsible, closer inspection of the regression lines was taken. While the regression plots were expected to have a slope close to 0.5, where the standard deviation was proportional to abundance in a manner similar to that expected for shot-noise limits, the slopes were approaching 1.0 instead. These investigations resulted in testing the removal of outliers using a z-score test to determine the effect on the ability to associate and discriminate the six isomers. No significant difference in results was observed (Appendix Tables A4.1 – A4.12). Secondly, further investigation of the regression data indicated that there may be two linear regions with two different slopes. The breakpoint was determined manually and the resulting slopes and y-intercepts from the new regression lines were input into a two-slope comparison version of the template and all comparisons were re-evaluated.

For the Month 1 collections of FIBF and FBF comparison spectra, association and discrimination were retained at the 99.9% confidence level. However, using the refined comparison method resulted in an increase in the number of discriminating ions, with the largest increase from 12 to 22 ions.

For the Month 2 collections, association and discrimination were also retained at the 99.9% confidence level. Once again, the use of the refined method to predict standard deviation resulted in a higher number of discriminating ions for many of the comparisons than the use of the original comparison method, with the largest increase being from 13 to 17 ions.

Finally, for the Month 3 collections, association was still possible using the refined comparison method at the 99.9% confidence level. An incorrect association between *m*- and *p*-

FIBF using the original comparison method was corrected to a discrimination with one ion responsible for discrimination (*m/z* 234) at the 99.9% confidence level. All other discriminations were possible at the 99.9% confidence level using the refined method, and many of the comparisons resulted in a higher number of discriminating ions.

Using the refined method to predict standard deviation resulted in similar overall association and discrimination results of the comparisons of the two sets of isomers. However, there was an increase in the number of discrimination ions in most comparisons as well as a reversal of an incorrect association to a correct discrimination. These results may increase the confidence in the discrimination power of the method and prove useful in a forensic laboratory setting. In order to utilize the refined method to predict standard deviation based on two regions of the regression, a more objective method of determining the breakpoint is necessary. Alternatively, instead of using a method to predict standard deviation that involves using two linear regions within a regression line, a more accurate equation which represents the additive variation of all three sources of noise within the election multiplier response should be investigated.

APPENDIX

Table A4.1 Comparisons of Month 1 FIBF comparison spectra and corresponding Month 1 FIBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	13	13	70, 71, 90, 102, 110, 111, 122, 130, 144, 149, 164, 165, 185	70, 71, 90, 102, 110, 111, 122, 130, 144, 149, 164, 165, 185
	p-FIBF	10	9	71, 84, 90, 111, 112, 130, 143, 144, 164, 165	71, 84, 90, 111, 112, 130, 144, 164, 165
***m*-FIBF**	*o*-FIBF	8	9	43, 71, 90, 95, 118, 148, 164, 165	43, 71, 90, 95, 102, 118, 148, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	2	2	84, 234	84, 234
***p*-FIBF**	*o*-FIBF	2	2	71, 164	71, 164
	m-FIBF	4	4	70, 110, 164, 234	70, 110, 164, 234
	p-FIBF	1	1	366	366

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.2 Comparisons of Month 1 FBF comparison spectra and corresponding Month 1 FIBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FIBF**	*o*-FBF	3	3	43, 113, 164	
	m-FBF	12	13	43, 44, 90, 105, 111, 122, 130, 144, 149, 164, 190, 208	43, 44, 90, 105, 111, 122, 130, 144, 149, 164, 190, 204, 208
	p-FBF	11	12	43, 44, 84, 90, 122, 130, 144, 176, 185, 208, 234	43, 44, 84, 90, 105, 122, 130, 144, 176, 185, 208, 234
***m*-FIBF**	*o*-FBF	14	13	43, 71, 90, 102, 110, 112, 113, 118, 143, 144, 149, 164, 165, 176	43, 71, 90, 102, 110, 112, 113, 118, 143, 144, 149, 164, 165,
	m-FBF	3	4	43, 71, 113	43, 71, 113, 164
	p-FBF	7	6	43, 71, 84, 164, 176, 234, 235	43, 71, 84, 164, 176, 234
***p*-FIBF**	*o*-FBF	19	17	43, 71, 72, 90, 95, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 159, 164, 165, 166	43, 71, 72, 90, 95, 102, 112, 113, 118, 124, 130, 136, 143, 144, 164, 165, 166
	m-FBF	6	5	43, 44, 71, 122, 149, 366	43, 44, 71, 122, 149
	p-FBF	3	3	43, 44, 71	43, 44, 71

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.3 Comparisons of Month 1 FBF comparison spectra and corresponding Month 1 FBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FBF**	*o*-FBF	0	1	–	43
	m-FBF	17	17	43, 44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 164, 165, 185	43, 44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 164, 165, 185
	p-FBF	14	13	43, 44, 71, 90, 95, 102, 118, 130, 143, 144, 164, 165, 234, 257	43, 44, 71, 90, 95, 102, 118, 130, 144, 164, 165, 234, 257
***m*-FBF**	*o*-FBF	16	16	44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 159, 164, 165	44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 159, 164, 165
	m-FBF	0	0	–	–
	p-FBF	1	1	234	234
***p*-FBF**	*o*-FBF	11	11	71, 90, 102, 118, 130, 143, 144, 159, 164, 165, 234	71, 90, 102, 118, 130, 143, 144, 159, 164, 165, 234
	m-FBF	3	3	164, 176, 234	164, 176, 234
	p-FBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.4 Comparisons of Month 1 FIBF comparison spectra and corresponding Month 1 FBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FBF**	*o*-FIBF	2	3	43, 71	43, 71, 164
	m-FIBF	19	18	43, 71, 90, 95, 102, 110, 111, 112, 113, 118, 124, 130, 143, 144, 148, 157, 164, 165, 257	43, 71, 90, 95, 102, 110, 111, 112, 113, 118, 124, 130, 143, 144, 148, 157, 164, 165
	p-FIBF	17	18	43, 71, 72, 90, 95, 102, 112, 113, 118, 124, 130, 138, 143, 144, 148, 164, 165	43, 71, 72, 90, 95, 102, 112, 113, 118, 124, 130, 136, 138, 143, 144, 148, 164, 165
***m*-FBF**	*o*-FIBF	6	6	43, 44, 122, 149, 164, 190	43, 44, 122, 149, 164, 190
	m-FIBF	4	4	43, 44, 113, 164	43, 44, 113, 164
	p-FIBF	4	4	43, 44, 112, 122	43, 44, 112, 122
***p*-FBF**	*o*-FIBF	5	5	43, 44, 122, 176, 234	43, 44, 122, 176, 234
	m-FIBF	8	8	43, 71, 110, 164, 165, 176, 234, 235	43, 71, 110, 164, 165, 176, 234, 235
	p-FIBF	5	5	43, 44, 71, 164, 176	43, 44, 71, 164, 176

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.5 Comparisons of Month 2 FIBF comparison spectra and corresponding Month 2 FIBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	8	8	43, 44, 71, 90, 102, 111, 148, 164	43, 44, 71, 90, 102, 111, 148, 164
	p-FIBF	5	5	71, 111, 112, 164, 234	71, 111, 112, 164, 234
***m*-FIBF**	*o*-FIBF	7	7	71, 90, 102, 118, 149, 164, 165	71, 90, 102, 118, 149, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	1	1	234	234
***p*-FIBF**	*o*-FIBF	5	7	71, 90, 130, 143, 164	71, 90, 130, 143, 144, 164, 165
	m-FIBF	5	5	43, 44, 71, 84, 234	43, 44, 71, 84, 234
	p-FIBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.6 Comparisons of Month 2 FBF comparison spectra and corresponding Month 2 FIBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FIBF**	*o*-FBF	4	5	44, 111, 113, 164	43, 44, 111, 113, 164
	m-FBF	7	9	44, 71, 95, 111, 118, 122, 148	43, 44, 71, 95, 102, 111, 118, 122, 148
	p-FBF	7	7	43, 44, 90, 102, 118, 176, 234	43, 44, 90, 102, 118, 176, 234
***m*-FIBF**	*o*-FBF	13	13	43, 90, 102, 110, 112, 113, 118, 136, 143, 144, 149, 164, 165	43, 90, 102, 110, 112, 113, 118, 136, 143, 144, 149, 164, 165
	m-FBF	3	3	43, 44, 164	43, 44, 164
	p-FBF	5	5	43, 71, 164, 176, 234	43, 71, 164, 176, 234
***p*-FIBF**	*o*-FBF	17	17	43, 44, 71, 90, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 150, 164, 165	43, 44, 71, 90, 102, 112, 113, 116, 118, 124, 130, 136, 143, 144, 150, 164, 165
	m-FBF	2	3	43, 44	43, 44, 124
	p-FBF	3	4	43, 44, 164	43, 44, 71, 164

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.7 Comparisons of Month 2 FBF comparison spectra and corresponding Month 2 FBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FBF**	*o*-FBF	0	0	–	–
	m-FBF	13	14	43, 44, 71, 90, 91, 95, 102, 118, 144, 148, 164, 165, 276	43, 44, 71, 90, 91, 95, 102, 118, 144, 148, 164, 165, 257, 276
	p-FBF	10	9	43, 44, 71, 90, 95, 102, 118, 144, 164, 234	43, 44, 90, 95, 102, 118, 144, 164, 234
***m*-FBF**	*o*-FBF	13	15	44, 71, 90, 102, 110, 118, 122, 130, 143, 144, 149, 164, 165	44, 70, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 149, 164, 165
	m-FBF	0	0	–	–
	p-FBF	1	1	234	234
***p*-FBF**	*o*-FBF	11	13	43, 44, 90, 130, 143, 144, 164, 176, 234	43, 44, 90, 130, 143, 144, 164, 176, 234
	m-FBF	1	1	234	234
	p-FBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.8 Comparisons of Month 2 FIBF comparison spectra and corresponding Month 2 FBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FBF**	*o*-FIBF	2	3	43, 71	43, 71, 105
	m-FIBF	12	13	43, 71, 90, 91, 102, 105, 110, 113, 118, 144, 164, 165	43, 44, 71, 90, 91, 102, 105, 110, 113, 118, 144, 164, 165
	p-FIBF	16	17	43, 71, 72, 90, 95, 102, 112, 113, 118, 143, 144, 148, 159, 164, 165, 276	43, 71, 72, 90, 91, 95, 102, 112, 113, 118, 143, 144, 148, 159, 164, 165, 276
***m*-FBF**	*o*-FIBF	9	9	43, 44, 90, 122, 130, 143, 144, 149, 164	43, 44, 90, 122, 130, 143, 144, 149, 164
	m-FIBF	2	3	43, 71	43, 71, 105
	p-FIBF	2	2	43, 44	43, 44
***p*-FBF**	*o*-FIBF	7	7	43, 44, 90, 130, 143, 176, 234	43, 44, 90, 130, 143, 176, 234
	m-FIBF	6	6	43, 71, 84, 164, 176, 234	43, 71, 84, 164, 176, 234
	p-FIBF	5	5	43, 44, 71, 164, 176	43, 44, 71, 164, 176

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.9 Comparisons of Month 3 FIBF comparison spectra and corresponding Month 3 FIBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FIBF**	*o*-FIBF	0	0	–	–
	m-FIBF	5	5	71, 90, 102, 164, 165	71, 90, 102, 164, 165
	p-FIBF	3	3	71, 95, 164	71, 95, 164
***m*-FIBF**	*o*-FIBF	7	7	71, 95, 102, 118, 149, 164, 165	71, 95, 102, 118, 149, 164, 165
	m-FIBF	0	0	–	–
	p-FIBF	0	0	–	–
***p*-FIBF**	*o*-FIBF	5	4	71, 90, 95, 144, 164	71, 90, 95, 164
	m-FIBF	2	2	164, 234	164, 234
	p-FIBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.10 Comparisons of Month 3 FBF comparison spectra and corresponding Month 3 FIBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FIBF**	*o*-FBF	2	2	43, 164	43, 164
	m-FBF	8	8	43, 44, 71, 93, 95, 122, 148, 164	43, 44, 71, 93, 95, 122, 148, 164
	p-FBF	3	3	43, 44, 234	43, 44, 234
***m*-FIBF**	*o*-FBF	6	6	43, 90, 102, 118, 164, 165	43, 90, 102, 118, 164, 165
	m-FBF	4	4	43, 44, 93, 164	43, 44, 93, 164
	p-FBF	3	3	43, 164, 234	43, 164, 234
***p*-FIBF**	*o*-FBF	14	13	43, 71, 90, 95, 102, 112, 118, 124, 130, 136, 143, 144, 164, 165	43, 71, 90, 95, 102, 112, 118, 124, 130, 136, 144, 164, 165
	m-FBF	2	2	43, 44	43, 44
	p-FBF	4	3	43, 44, 111, 164	43, 44, 164

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.11 Comparisons of Month 3 FBF comparison spectra and corresponding Month 3 FBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FBF**	*o*-FBF	0	1	–	43
	m-FBF	12	11	43, 44, 71, 90, 102, 118, 122, 144, 148, 165, 171	43, 44, 90, 102, 118, 122, 144, 148, 165, 171
	p-FBF	3	2	118, 164, 171	118, 164
***m*-FBF**	*o*-FBF	11	10	90, 102, 110, 118, 122, 130, 144, 149, 164, 165, 171	90, 102, 118, 122, 130, 144, 149, 164, 165, 171
	m-FBF	1	1	93	93
	p-FBF	0	0	–	–
***p*-FBF**	*o*-FBF	7	6	90, 102, 118, 130, 144, 164, 171	90, 102, 118, 130, 164, 171
	m-FBF	3	3	43, 93, 234	43, 93, 234
	p-FBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.12 Comparisons of Month 3 FIBF comparison spectra and corresponding Month 3 FBF reference spectra at the 99.9% confidence level before and after the removal of outliers

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Outliers Removed	Original	Outliers Removed
***o*-FBF**	*o*-FIBF	2	2	43, 164	43, 164
	m-FIBF	8	8	43, 90, 102, 110, 144, 164, 165, 171	43, 90, 102, 110, 144, 164, 165, 171
	p-FIBF	15	15	43, 71, 90, 91, 95, 102, 112, 113, 118, 124, 130, 143, 144, 164, 165	43, 71, 90, 91, 95, 102, 112, 113, 118, 124, 130, 143, 144, 164, 165
***m*-FBF**	*o*-FIBF	10	10	43, 44, 71, 90, 95, 102, 118, 122, 149, 164	43, 44, 71, 90, 95, 102, 118, 122, 149, 164
	m-FIBF	2	2	43, 164	43, 164
	p-FIBF	3	3	43, 44, 149	43, 44, 149
***p*-FBF**	*o*-FIBF	8	8	43, 44, 90, 102, 118, 130, 176, 234	43, 44, 90, 102, 118, 130, 176, 234
	m-FIBF	5	5	43, 71, 164, 176, 234	43, 71, 164, 176, 234
	p-FIBF	4	4	43, 44, 71, 164	43, 44, 71, 164

Table A4.13 Comparisons of Month 1A FIBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FIBF	1	4	43	43, 55, 71, 91
	m-FIBF	9	13	43, 71, 90, 102, 118, 144, 164, 165, 171	43, 44, 71, 90, 102, 110, 112, 118, 144, 149, 164, 165, 171
	p-FIBF	15	21	43, 71, 90, 95, 102, 112, 113, 118, 124, 141, 143, 144, 164, 165, 166	43, 71, 90, 91, 95, 102, 112, 113, 118, 124, 130, 136, 141, 143, 144, 148, 164, 165, 166, 171, 178
***m*-FBF**	*o*-FIBF	4	5	43, 122, 149, 164	43, 122, 148, 149, 164
	m-FIBF	3	4	43, 71, 164	43, 71, 164, 165
	p-FIBF	4	5	43, 44, 112, 141	43, 44, 112, 122, 141
***p*-FBF**	*o*-FIBF	5	9	43, 111, 130, 176, 234	43, 51, 84, 94, 111, 122, 130, 176, 234
	m-FIBF	6	7	43, 71, 164, 165, 176, 234	43, 71, 164, 165, 176, 234, 235
	p-FIBF	4	4	43, 141, 164, 176	43, 141, 164, 176

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.14 Comparisons of Month 1B FBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FBF	0	0	–	–
	m-FBF	17	20	43, 44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 164, 165, 185	43, 44, 71, 72, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 162, 164, 165, 185, 214
	p-FBF	14	15	43, 44, 71, 90, 95, 102, 118, 130, 143, 144, 164, 165, 234, 257	43, 44, 71, 90, 95, 102, 118, 130, 143, 144, 164, 165, 234, 257
***m*-FBF**	*o*-FBF	16	23	44, 71, 90, 95, 102, 110, 118, 122, 130, 143, 144, 148, 149, 159, 164, 165	44, 71, 90, 95, 102, 109, 110, 111, 116, 118, 122, 128, 130, 143, 144, 148, 149, 159, 160, 164, 165, 190, 214
	m-FBF	0	0	–	–
	p-FBF	1	2	234	84, 234
***p*-FBF**	*o*-FBF	11	15	71, 90, 102, 118, 130, 143, 144, 159, 164, 165, 234	71, 90, 102, 112, 118, 122, 130, 143, 144, 159, 164, 165, 176, 234, 257
	m-FBF	3	3	164, 176, 234	164, 176, 234
	p-FBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.15 Comparisons of Month 1B FIBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FIBF	2	3	43, 71	43, 71, 205
	m-FIBF	19	25	43, 71, 90, 95, 102, 110, 111, 112, 113, 118, 124, 130, 143, 144, 148, 157, 164, 165, 257	43, 71, 90, 95, 102, 110, 111, 112, 113, 118, 122, 124, 130, 132, 136, 143, 144, 148, 157, 164, 165, 166, 171, 176, 257
	p-FIBF	17	23	43, 71, 72, 90, 95, 102, 112, 113, 118, 124, 130, 138, 143, 144, 148, 164, 165	43, 71, 72, 90, 91, 95, 102, 110, 111, 112, 113, 118, 124, 130, 136, 138, 143, 144, 148, 164, 165, 185, 214
***m*-FBF**	*o*-FIBF	6	13	43, 44, 122, 149, 164, 190	43, 44, 95, 122, 130, 144, 148, 149, 164, 190, 205, 208, 247
	m-FIBF	4	7	43, 44, 113, 164	43, 44, 71, 113, 164, 165, 176
	p-FIBF	4	5	43, 44, 112, 122	43, 44, 112, 122, 149

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.15 (cont'd.)

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***p*-FBF**	*o*-FIBF	5	12	43, 44, 122, 176, 234	43, 44, 84, 90, 122, 130, 176, 205, 208, 234, 235, 248
	m-FIBF	8	10	43, 71, 110, 164, 165, 176, 234, 235	43, 70, 71, 84, 110, 164, 165, 176, 234, 235
	p-FIBF	5	7	43, 44, 71, 164, 176	43, 44, 71, 98, 112, 164, 176

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.16 Comparisons of Month 2 FBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FBF	0	0	–	–
	m-FBF	13	14	43, 44, 71, 90, 91, 95, 102, 118, 144, 148, 164, 165, 276	43, 44, 71, 90, 91, 95, 102, 118, 144, 148, 164, 165, 257, 276
	p-FBF	10	12	43, 44, 71, 90, 95, 102, 118, 144, 164, 234	43, 44, 71, 90, 95, 102, 118, 144, 164, 176, 234, 276
***m*-FBF**	*o*-FBF	13	16	44, 71, 90, 102, 110, 118, 122, 130, 143, 144, 149, 164, 165	44, 71, 90, 102, 110, 112, 116, 118, 122, 130, 143, 144, 149, 164, 165, 214
	m-FBF	0	0	–	–
	p-FBF	1	1	234	234
***p*-FBF**	*o*-FBF	11	14	43, 44, 90, 130, 143, 144, 164, 176, 234	44, 71, 90, 95, 102, 112, 118, 130, 143, 144, 164, 176, 214, 234
	m-FBF	1	3	234	84, 176, 234
	p-FBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.17 Comparisons of Month 2 FIBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FIBF	2	2	43, 71	43, 71
	m-FIBF	12	14	43, 71, 90, 91, 102, 105, 110, 113, 118, 144, 164, 165	43, 71, 84, 90, 91, 102, 105, 110, 113, 118, 144, 164, 165, 171
	p-FIBF	16	16	43, 71, 72, 90, 95, 102, 112, 113, 118, 143, 144, 148, 159, 164, 165, 276	43, 71, 72, 90, 95, 102, 112, 113, 118, 143, 144, 148, 159, 164, 165, 276
***m*-FBF**	*o*-FIBF	9	10	43, 44, 90, 122, 130, 143, 144, 149, 164	43, 44, 90, 118, 122, 130, 143, 144, 149, 164
	m-FIBF	2	2	43, 71	43, 71
	p-FIBF	2	2	43, 44	43, 44
***p*-FBF**	*o*-FIBF	7	9	43, 44, 90, 130, 143, 176, 234	43, 44, 84, 90, 118, 130, 143, 176, 234
	m-FIBF	6	7	43, 71, 84, 164, 176, 234	43, 71, 84, 164, 176, 234, 235
	p-FIBF	5	5	43, 44, 71, 164, 176	43, 44, 71, 164, 176

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.18 Comparisons of Month 3 FBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	**Comparison Spectrum**	**Number of Discriminating Ions**		***m/z* Values of Discriminating Ions**	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FBF	0	0	–	–
	m-FBF	12	13	43, 44, 71, 90, 102, 118, 122, 144, 148, 165, 171	43, 44, 71, 90, 95, 102, 118, 122, 144, 148, 165, 171
	p-FBF	3	2	118, 164, 171	118, 164
***m*-FBF**	*o*-FBF	11	12	90, 102, 110, 118, 122, 130, 144, 149, 164, 165, 171	44, 90, 102, 110, 118, 122, 130, 144, 149, 164, 165, 171
	m-FBF	1	2	93	93, 121
	p-FBF	0	0	–	–
***p*-FBF**	*o*-FBF	7	7	90, 102, 118, 130, 144, 164, 171	90, 102, 118, 130, 144, 164, 171
	m-FBF	3	3	43, 93, 234	43, 93, 234
	p-FBF	0	0	–	–

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

Table A4.19 Comparisons of Month 3 FIBF comparison spectra and corresponding FBF reference spectra at the 99.9% confidence level using the original comparison method and the refined method of standard deviation prediction

Reference Spectrum	Comparison Spectrum	Number of Discriminating Ions		*m/z* Values of Discriminating Ions	
		Original	Refined	Original	Refined
***o*-FBF**	*o*-FIBF	2	2	43, 164	43, 164
	m-FIBF	8	9	43, 90, 102, 110, 144, 164, 165, 171	43, 90, 102, 110, 136, 144, 164, 165, 171
	p-FIBF	15	15	43, 71, 90, 91, 95, 102, 112, 113, 118, 124, 130, 143, 144, 164, 165	43, 71, 90, 91, 95, 102, 112, 113, 118, 124, 130, 143, 144, 164, 165
***m*-FBF**	*o*-FIBF	10	11	43, 44, 71, 90, 95, 102, 118, 122, 149, 164	43, 44, 71, 90, 95, 102, 118, 122, 130, 149, 164
	m-FIBF	2	2	43, 164	43, 164
	p-FIBF	3	4	43, 44, 149	43, 44, 122, 149
***p*-FBF**	*o*-FIBF	8	9	43, 44, 90, 102, 118, 130, 176, 234	43, 44, 90, 102, 118, 122, 130, 176, 234
	m-FIBF	5	6	43, 71, 164, 176, 234	43, 71, 164, 165, 176, 234
	p-FIBF	4	4	43, 44, 71, 164	43, 44, 71, 164

*Ions in red denote additional discriminating ions that were only identified in the refined comparisons

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V. Conclusions and Future Work

5.1 Conclusions

The overall objective in this research was to investigate the robustness of the previously developed statistical comparison method to differentiate positional isomers using mass spectral data. Mass spectra of two sets of fentanyl isomers, fluoroisobutyryl fentanyl (FIBF) and fluorobutyryl fentanyl (FBF), collected during a three-month period, were used in this work. First, association and discrimination of the isomers within and between each set was investigated with mass spectra collected in the first month of analysis. Spectra of the FIBF positional isomers and the FIBF and FBF structural isomers were correctly associated and discriminated, mostly at the 99.9% confidence level, with only three exceptions. During the three-month study, the effects of major instrument maintenance (involving venting of the system) as well as high instrument usage (involving other research groups using the same instrument for a multitude of purposes) on the ability to maintain proper association and discrimination of the fentanyl isomers were investigated. Throughout the three months, the overall success of appropriate association and discrimination was maintained at the 99.9% confidence level, with small differences in the number and *m/z* value of discriminating ions. Nonetheless, certain ions were identified as reliable ions for the discrimination between the positional isomers of FIBF and the structural isomers of FIBF and FBF.

In addition, the method used to predict standard deviation based on modeling the electron multiplier response was further refined by testing the effects of removing outliers and using two-slopes to better describe the data. While removing outliers had a negligible effect on the outcomes of the statistical comparisons, the investigation into using two separate slopes to more accurately model the electron multiplier response proved to positively impact the statistical comparisons. One incorrect association (false positive) was reversed to a discrimination and many of the discriminating comparisons resulted in a greater number of discriminating ions, giving more confidence in the distinction of the fentanyl isomers.

This user-friendly and rapid statistical comparison method can be implemented into forensic laboratories to analyze submitted samples using mass spectral data that are already routinely collected. Typically, a forensic analyst will analyze a submitted sample using gas chromatography-mass spectrometry (GC-MS) and compare the resulting mass spectrum to a spectral library within the analysis software. Using the initial identification from the mass spectral library, a reference standard can be analyzed, and the data can be visually compared. In additional to the visual comparison, the mass spectra of the submitted sample and that of the reference standard could be entered into the statistical comparison method to determine if the two spectra are statistically distinguishable or indistinguishable from one another. Using this method, in addition to a visual assessment of the spectra, allows for statistical confidence in the identification of the submitted sample. And, in cases where the two spectra are statistically different, the ions responsible for identification can be identified.

5.2 Future Work

This work focused on demonstrating the ability of the statistical comparison method to discriminate two sets of fentanyl isomers: FIBF and FBF positional isomers. The method has also been applied to successfully discriminate positional isomers of ethylmethcathinone (EMC) and fluoromethamphetamine (FMA).[1] Given the increase in submissions of novel psychoactive substances (NPS) and related isomers in forensic laboratories, further investigation into the robustness of the method is warranted. Fentanyl and NPS analogs are a growing problem in the

United States and are expected to remain a serious threat in the years to come.[2] Therefore, the ability to identify a wide range of positional isomers using instrumentation that is readily available in laboratories would be an advantage. In order to obtain that statistical confidence, the statistical comparison method must be tested on a wide range of NPS analogs.

In order to truly refine the method of standard deviation prediction, a new method to model the electron multiplier response should be investigated. Instead of automating a method to separate the regression line into two linear regions, using a curve to represent the three sources of noise (background, shot, and proportional) would be more accurate and potentially lead to more accurate comparison results.

Additionally, a true concentration study should be performed to determine the ability of the statistical comparison method to discriminate isomers at various concentrations. Previous research indicates that lower concentration of sample results in fewer discriminating ions present; therefore, a full concentration study would allow the determination of optimal concentrations to be used for statistical comparisons. A more in depth concentration study would also allow the determination of concentrations below the threshold for accurate association and discrimination.[1]

Finally, the statistical comparison method should be applied to blind samples and case work samples to further evaluate the robustness and ruggedness of the method. This test would be a true determination of how successfully the statistical comparison method could be applied to forensic case work.

Through these additional studies, as well as further refinement of the method of standard deviation, continued evaluation of the robustness of the statistical comparison method is possible. While these steps are needed, this work has demonstrated the ability of the method to successfully discriminate between two sets of structural and positional isomers with a high degree of spectral similarity. By testing the method on fentanyl isomers, the method was applied to a new range of NPS compounds that have proven to be difficult to discriminate using other methods. Additionally, the preliminary study into the effects of refining the method of standard deviation prediction on the association and discrimination demonstrated the potential effectiveness of utilizing a segmented regression analysis to increase the statistical confidence in discriminating power.

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